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**GENETIC AND ENVIRONMENTAL DETERMINANTS OF
PAGET'S DISEASE OF BONE**

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A thesis presented to the School of Medicine & Veterinary Medicine

Department of Molecular and Cellular Biology, University of Edinburgh in candidature for the degree
of Doctor of Philosophy in Medicine

Supervisor: Professor Stuart H. Ralston

And

Dr. Kirsteen Goodman

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To my family

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Declaration

I hereby declare that this thesis has been composed by me and that it has not been accepted in any previous application for a degree. The work, of which it is a record, except where specifically acknowledged, has been carried out by the author. The information obtained from other sources rather than this study has been specifically acknowledged by means of reference.

Micaela Rios

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Publications, Presentations and awards

Publications related to this thesis

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- Visconti, M. Rios., Langston, A. L., Goodman K., Selby, P. L., Fraser, W. D., Ralston, S. H. "Relation between circulation measles virus antibodies and disease severity in Paget's disease: The prism study". Bone, vol 48, pp. S241. Bone 2011. **Abstract for the ECTS : Athens 2011.**
- Albagha, O. M. E., Wani, S. E., Visconti, M. R., Alonso, N., Goodman, K., Brandi, M. L., Cundy, T., Chung, P. Y. J., Dargie, R., Devogelaer, J. P., Falchetti, A., Fraser, W. D., Gennari, L., Gianfrancesco, F., Hooper, M. J., Van Hul, W., Isaia, G., Nicholson, G. C., Nuti, R., Papapoulos, S., Montes, J. D. P., Ratajczak, T., Rea, S. L., Rendina, D., Gonzalez-Sarmiento, R., di Stefano, M., Ward, L. C., Walsh, J. P., & Ralston, S. H. 2011, "Genome-wide association identifies three new susceptibility loci for Paget's disease of bone", *Nature Genetics*, vol. 43, no. 7, p. 685-U104.
- Albagha, O. M. E., Visconti, M. R., Alonso, N., Langston, A. L., Cundy, T., Dargie, R., Dunlop, M. G., Fraser, W. D., Hooper, M. J., Isaia, G., Nicholson, G. C., Montes, J. D., Gonzalez-Sarmiento, R., di Stefano, M., Tenesa, A., Walsh, J. P., & Ralston, S. H. 2010, "Genome-wide association study identifies variants at CSF1, OPTN and TNFRSF11A as genetic risk factors for Paget's disease of bone", *Nature Genetics*, vol. 42, no. 6, p. 520-U26.
- Visconti, M. R., Langston, A. L., Alonso, N., Goodman, K., Selby, P. L., Fraser, W. D., & Ralston, S. H. 2010, "Mutations of *SQSTM1* Are Associated With Severity and Clinical Outcome in Paget Disease of Bone", *Journal of Bone and Mineral Research*, vol. 25, no. 11, pp. 2368-2373.

Presentations

- ❖ 3rd Joint meeting European Calcified Tissues Society & International Bone and Mineral Society 6-11 May 2011. “Relation Between Circulation Measles virus antibodies and disease severity in Paget’s Disease: The PRISM study”. M. Rios Visconti, Omar M.E. Albagha, Kirsteen Goodman, Peter L Selby, William D Fraser, Stuart H Ralston. Poster presentation.
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Awards

- ❖ ECTS 35th European Symposium on Calcified Tissues 24-28 May 2008. **ECTS New Investigator Award.**

Abbreviations

°C	degrees Celsius
μl	microliters
μM	micromolar
1,25D ₃	1, 25 dihydroxyvitamin D ₃
A	adenine
AID	acidic interaction domain
AKt	serine/ threonine protein kinase
ALP	alkaline phosphatase
ANOVA	analysis of variance between groups
AP-1	activator protein-1
aPKC	atypical protein kinase C
Arg	arginine
ATF4	activating transcription factor 4.
ATP	adenosine triphosphate.
Atp6v0d2	ATPase, H ⁺ transporting, lysosomal 38kDa, V0 subunit d2.
BAD	Bcl-2- associated death promoter.
Bcl-2	B cell lymphoma 2.
BFU	bone forming unit.
BMPs	bone morphogenetic proteins.
bp	base-pairs.
BRU	bone remodelling unit.
Bsp	bone sialo-protein.
C	cytosine.
catK	cathepsin K.
Cbfa1	core-binding factor α-1.
cDNA	complementary DNA.
CDV	canine distemper virus.
c-fos	cellular proto-oncogene protein.
CFU-GM	granulocyte-macrophage colony stimulating factor.
CSF1	colony stimulation factor 1
c-src	cellular sarcoma protein.

c-jun	cellular (nuclear) retrovirus associated DNA sequence, protein.
DC-STAMP	dendritic cell-specific transmembrane protein.
ddATP	dideoxy adenine triphosphate.
ddCTP	dideoxy cytosine triphosphate.
ddGTP	dideoxy guanine triphosphate.
ddTTP	dideoxy thymine triphosphate.
DNA	deoxyribonucleic acid.
dNTP	deoxy N-triphosphate.
ELISA	enzyme linked immunosorbent assay.
ENT	ear, nose and throat.
EphB2	ephrinB2
EphB4	ephrin B4
ESH	expansile skeletal hyperphosphatasia.
FEO	familial expansile osteolysis
FGF	fibroblast growth factor
FZD	frizzled G protein
G	gram
G	guanine
Gln	glycine
Glu	glutamine
HDMP	hydroxymethylene diphosphonate
HLA	human leukocyte antigen
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IFN- γ	interferon γ
IGF	insulin-like growth factor
I κ B	inhibitor of NF- κ B
IKK	I κ B kinase
IL-1	interleukin-1
IL-6	interleukin-6
INF β	interferon beta
INF γ	interferon gamma
JNK	protein Janus kinase

Kb	kilobases
kDa	kilodaltons
L	litres
LDL	low density lipoprotein
LOD	logarithm of odds ratio
LOH	loss of heterozygosity
LPA	lysophosphatidic acid
LRP	interleukin-1
LRP5	low density lipoprotein receptor related protein 5.
M	molar
M-CSF	macrophage-colony stimulating factor
MgCl ₂	magnesium chloride
mg	milligrams
Mitf	microphthalmia associated transcription factor
MKP-Mmap	kinase phosphatase from macrophages
ml	millilitres
mM	millimolar
MRZ	MRZ reaction.
mRNA	messenger RNA
MV	measles virus
NF-κB	nuclear factor κB
NFAT	nuclear factor of activated T cells
ng	nanogrammes
NIK	NF-κB-inducing kinase
NUP205	nucleoporin 205 KDa gene
Ocn	osteocalcin
OPG	osteoprotegerin
OPGL	osteoprotegerin ligand
OPTN	optineurin
OSX	osterix
PCR	polymerase chain reaction
PDB	Paget's Disease of Bone
PDGF	platelet-derived growth factor
PEST	proline/glutamic acid/serine/threonine sequence or domain

PML	promyelocytic leukemia gen
PTH	parathyroid hormone
PU-1	Protein that in humans is encoded by the <i>SP11</i> gene.
RANK	receptor-activator of NF- κ B
RANKL	receptor-activator of nuclear factor κ B ligand
RFLP	restriction fragment length polymorphism
RIN3	member of the RIN family, Ras and Ran interactor 3
RNA	ribonucleic acid
ROS	reactive oxygen species
RSV	respiratory syncytial virus
RT-PCR	reverse transcription protein chain reaction
Runx2	runt-related transcription factor 2
SAP	shrimp alkaline phosphatase
SEM	standard error of the mean. SEM= SD/SQRT (number)
Ser	serine
SD	standard deviation
SH	src-homology domain
SNP	single nucleotide polymorphism
SQRT	square root of the population size
<i>SQSTM1</i>	sequestosome 1
Sts	sequence-tagged site
T	thymine
TAB2	TAK1-binding protein-2
TAK1	TRAF6-associated kinase-1
Taq	<i>Thermus aquaticus</i>
-ter	terminus
TGF- β	transforming growth factor beta.
TM7SF4	transmembrane 7 superfamily member 4.
TNF α	tumour necrosis factor α
TNFRSF11A	tumour necrosis factor receptor superfamily member 11A
TRAcP	tartrate resistant acid phosphatase
TRAF	TNF receptor-associated factor
UBA	ubiquitin-associated domain
VZV	varicella-zoster virus

VEGF	vascular endothelial growth factor
Wnt	hybrid name from Wg (wingless) in Drosophila and Integration 1 in dropsophila, best Wnt described.

ABSTRACT

Genetic factors play an important role in the pathogenesis of Paget's Disease of Bone (PDB). The most important predisposing gene is *SQSTM1* which is mutated in about 10% of patients, additionally common variants at seven other loci have also been shown to predispose to PDB as well as environmental factors which are also important in the pathogenesis of PDB. Little research has been conducted on the relationship between the genetic variants that predispose to PDB and disease severity. Similarly, only limited information exists on the role that gene-environment interactions play in the pathogenesis of PDB or its severity. The aim of the present thesis was to explore these issues in participants of the Paget's Disease Randomised Trial of Intensive versus Symptomatic Management study (PRISM) and other study cohorts. In chapter 3, I investigate the relationship between *SQSTM1* mutation status, disease severity and clinical outcome in 737 patients from the PRISM study. Mutations of *SQSTM1* were detected in 80/737 (10.9%) patients. Mutation carriers had an earlier age at diagnosis; a greater number of affected bones and more commonly had required orthopaedic surgery and bisphosphonate therapy than those without mutations. Quality of life was significantly reduced in carriers and during the study; fractures were more common although most of these occurred in unaffected bone. This study demonstrates that *SQSTM1* mutations are strongly associated with disease severity and complications of PDB. In chapter 4, I study associations between common genetic variants identified by genome wide association (GWAS), clinical severity and extent of PDB, alone and in combination with *SQSTM1* mutations. This showed that these common variants were also associated with severity and extent of PDB in PRISM, but with weaker effects than *SQSTM1* mutations. The findings were replicated in a multinational study involving 1940 subjects from centres in Italy, Spain and Australia. In all cohorts the GWAS risk alleles acted in an additive manner with *SQSTM1* mutations to regulate disease severity and extent. By combining information from *SQSTM1* status and the new risk alleles, however, we are able to develop a genetic risk score which delineated three distinct groups with markedly differing effects on disease extent and severity.

In chapter 5, I study associations between PDB, severity and extent in relation to circulating levels of IgG antibodies against various viruses including Rubella, respiratory syncytial virus, distemper, varicella zoster virus, measles and mumps. We found little evidence of an

interaction between viral antibody titres and SQSTM1 in predicting disease severity with the notable exception of mumps virus where subjects with the highest levels of antibodies that were SQSTM1 positive had an increased age at diagnosis than the other genotype / viral antibody groups. Overall the studies do provide no support for the notion that patients with PDB have an abnormal antibody response to paramyxovirus or have had previous infections with these viruses more frequently than controls. This of course does not exclude the possibility that PDB patients might have a clinically occult slow virus infection which is not accompanied by an abnormality in the immune response. . This raises the possibility that genetic testing may be of value in identifying individuals at risk of developing severe disease and those at risk of complications. I also demonstrate that PBD patients have abnormalities in circulating antibodies to various viruses suggesting that the disease may be associated with disturbance in the response of the immune system to infectious agents but further investigation is required. This, perhaps, could explain the changes in the severity and prevalence of PDB that have been observed over recent years in several countries.

Chapter 1: Introduction

1.1. Bone

The function of the bone is to facilitate body movement, to support and protect internal organs of the body, to act as a site for haematopoiesis and to act as storage and a reservoir of minerals and ions for the maintenance of calcium and phosphate homeostasis.

Bone is a specialized type of connective tissue, which is composed of cells, vessels and extra cellular matrix components, which become mineralised with calcium salts in the form of hydroxyapatite. The bones also are covered with a thin layer of connective tissue called the periosteum and the ends of long bones are covered by articular cartilage. The external or periosteal surface of the bone is fibrous and functions as an anchor for the attachment of muscles, tendons and ligaments. Some bones (in adults is found only in the vertebrae, hips, breastbone, ribs, skull and at the ends) contain haematopoietic tissue in the bone marrow space.

Bones are composed of an outer shell of cortical (compact) bone, which surrounds a network of trabecular (plates) or cancellous bone (Fig.1.1) (Boyce et al. 2003). Cortical bone is dense, and accounts for 80% of the total bone mass in the adult skeleton. Cortical bone is most abundant in the shafts of long bones (Dempster et al. 2006). It has a slow turnover rate, when compared with trabecular bone and a high resistance to bending and torsion.

Cortical bone is composed of structures called osteons or Haversian systems. Each osteon consist of a central canal, containing vessels, nerves and possibly lymphatics. Osteons consist of concentric layers of mineralized bone.

Trabecular bone, consists of rod-and plate-like elements; joined together to form a spongy structure, which contains space for vessels and bone marrow. Trabecular bone is most abundant in vertebrae, pelvis, flat bones and the ends of the long bones. Trabecular bone

accounts for the remaining 20% of the total bone mass in adults, but contains nearly ten times the surface of the compact bone.

While not all the bones are equal in size and consistency, on average they comprise about 25% water, 45% of mineral and 30% organic matter, mainly collagen and other proteins.

The principal protein of the bone matrix is Type I collagen (Lammi et al. 2006), which is a fibrillar protein consisting of three individual protein chains wound together in a triple helix. Other proteins found in bone matrix include glycosaminoglycans, osteocalcin, osteonectin, bonesialo protein and osteopontin. Bone matrix confers properties of elasticity and flexibility and plays a role in its structural organization of the skeleton. Added to all the functions mentioned above, bones play a central role in calcium and phosphate metabolism, about 99% of all calcium in the body is in the bones. Stable levels of calcium in blood are essential to life, and bones act as source of calcium, releasing it or taking it up in response to regulatory hormones as PTH and 1, 25-dihydroxyvitamin D₃ (Dempster et al. 2006).

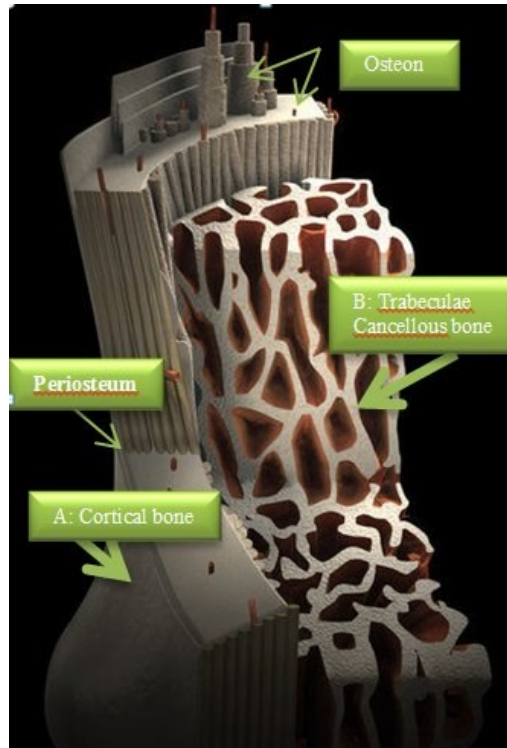


Figure.1.1: The image is showing the internal structure of bones. A; cortical bone, is dense and forms the surface of bones, while B: Trabecular or cancellous bones is spongy and makes up the bulk of their interior. Original image taken from: <http://www.sciencephoto.com/media/198137>.

The main minerals that can be found in bone are calcium, magnesium, sodium, and potassium. Heavy metals such lead, barium, uranium and strontium (Burton et al. 2003; Garcia-Sanchez et al. 2005; Jaworowski et al. 1985) are also present in low amounts. Bone and minerals are constantly being removed and replaced in a process known as bone remodelling (Horvath 2006).

The majority of the bones are formed during embryonic development by endochondral ossification, in which they are first modelled from an anlage of cartilage (Karsenty and Wagner 2002). Blood vessels invade the cartilage, which is resorbed by chondroclast, to form a medullary cavity. Subsequently the cartilage is replaced by cortical bone formed by periosteal apposition and by trabecular bone, which is laid down within the medullary space at the epiphyseal growth plate.

In the active skeleton, the integrity of bone is compromised by micro-fractures, excessive exercise or lack of exercise and others, but integrity of bone is maintained by the process of bone remodelling.

1.1.1. Bone remodelling

Bone in the adult skeleton is constantly being renewed and repaired in response to a variety of stimuli, through the process of bone remodelling. Bone remodelling can be divided into four stages, resorption, reversal, formation and quiescence (Fig. 1.2). During the resorption phase, osteoclast precursors are recruited to a specific resorption site where they differentiate to form mature osteoclasts. Following retraction of the bone-lining cells, osteoclasts attach to the bone surface by forming a sealing zone and create a greatly invaginated secretory surface called the ruffled border. The ruffled border creates an acidic and proteolytic environment which results in an active bone resorption site in the enclosed space, called a resorption pit or Howship's Lacuna. After osteoclasts have resorbed bone, there is a reversal phase during which the osteoclast undergoes apoptosis. Osteoblast precursors are attracted to the resorption pit, where they differentiate into mature osteoblast and secrete and deposit bone matrix. As bone formation proceeds, some osteoblast become embedded in the bone matrix and differentiate to osteocytes. Following bone formation, the osteoid is slowly mineralised to form mature bone. This is followed by a period of quiescence.

Under normal conditions the amount of bone removed by osteoclasts exactly equals the amount of bone formed by osteoblast. An imbalance in the regulation of bone remodelling, can result in bone diseases such osteoporosis, sclerosing bone disease, etc. In Paget's disease for example, there is up-regulation of bone resorption and bone formation, leading to production of abnormal bone with reduced strength.

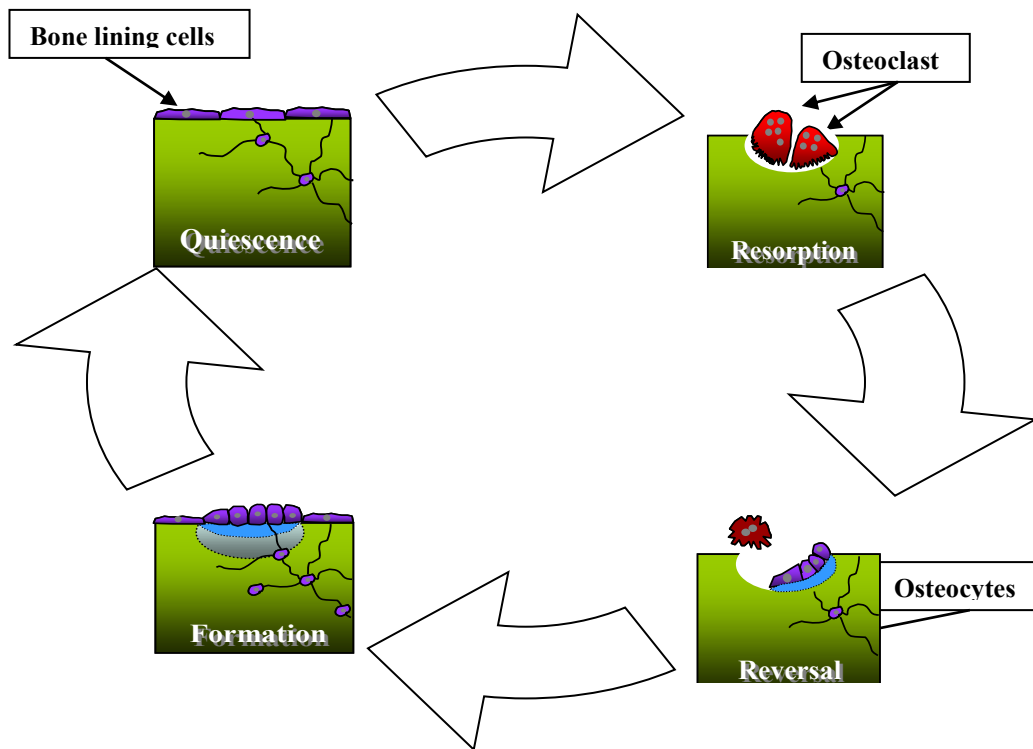


Figure.1.2. Bone remodelling.

Relative rates of bone resorption and bone formation relies on complex signalling pathways. These signalling pathways that regulate bone remodelling include several hormones, such as parathyroid hormone (PTH), vitamin D, growth hormone, cortico-steroids, sex-steroids, calcitonin, and several locally produced cytokines and growth factors (summary in Table 1.1).

Semaphorins are a big family of guidance molecules, secreted, transmembrane, and GPI-linked proteins, that have important roles in a variety of tissues. Recently published work, highlight the role of semaphorins on bone remodelling by a distinct mechanism, where Semaphorin 4d expressed by osteoclasts inhibits bone formation; semaphorin 3A exerts an osteoprotective effect by suppressing bone resorption and increasing bone formation (Takayanagi 2012). Also we must mention the role of ephrin ligands and receptors, where Eph4 signaling into osteoblast enhances differentiation and EphB2 signalling into osteoclast precursors suppresses osteoclast differentiation, inducing a shift from bone resorption to bone formation (Cheng et al. 2013; Jin et al. 2013; Zhao et al. 2006)

Systemic hormones such as parathyroid hormone (PTH), vitamin D3 and sex hormones affect bone remodelling throughout the skeleton and are responsible for increasing or decreasing bone remodelling. As an example, PTH, promotes RANKL production by osteoblast/stromal cells which accounts in part for the increased osteoclast activity seen in primary hyperparathyroidism (Boyce et al. 2003; Suda et al. 1999). In addition to stimulating bone resorption, PTH also has anabolic effects on bone through a stimulatory effect as bone formation (Rubin and Bilezikian 2003). This is associated with intermittent, rather than continuous, exposure to PTH, which stimulates bone formation more than resorption. Intermittent doses of PTH stimulate bone formation and reduce fracture risk in postmenopausal women with osteoporosis.

BONE RESORPTION		BONE FORMATION	
STIMULATES	INHIBITS	STIMULATES	INHIBITS
PTH	Oestrogen	PTH	IL-1
1,25-(OH) ₂ D ₃	Androgens	Oestrogen	TNF
IL-1	Calcitonin	Wnt	SOST
IL-6	IFN- γ	BMP	Semaphorin4d
PGE ₂	OPG	IGF	
TNF	Semaphorin3a	TGF- β	
M-CSF	EphB2	PGE ₂	
RANKL		Semaphorin3a	
TGF- β		EphrinB4	

Table 1.1: Main systemic and local factors involved in the regulation of bone remodelling; systemic hormones, local cytokines and growth factors.

Mechanical loading is also thought to influence local bone remodelling, by regulatory secretion of osteotropic factors by the osteocytes, most notably sclerostin (SOST) and RANKL (Keller and Kneissel 2005). The osteocytes work as true mechanotransducer being able to convert the perceived mechanical strain signal into a biological out-put . In primary osteocytes, fluid flow shear stress increases intracellular calcium; promotes the release of nitric oxide (NO), ATP, and prostaglandins; induces opening of connexin 43 hemichannels; and enhances gap junction functions. It has also been shown to induce bending of cilia and to initiate

signaling pathways such as the Wnt/ β -catenin and protein kinase A (PKA) pathways. The fluid flow shear stress also protects osteocytes against apoptosis, activates gene transcription and translation, and promotes dendrite elongation. It is still not clear what type of fluid flow shear stress occurs physiologically within the lacunocanalicular system (Dallas et al. 2013).

1.1.2. Osteocytes

Bone remodelling is carried out by osteoblasts, osteocytes and osteoclasts, which are called collectively the bone remodelling unit (BRU), coupled together via paracrine cell signalling (Dempster et al. 2006) (Fig. 1. 3).

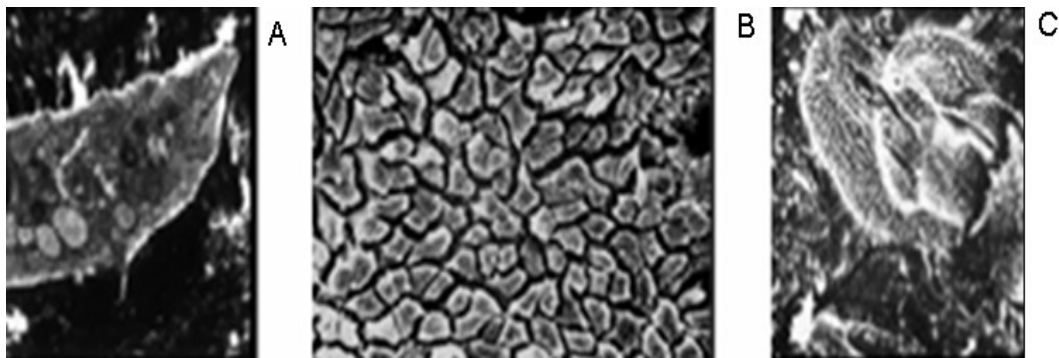


Fig.1.3. Bone Remodelling Unit: A. Transmission electron micrograph of a rat osteocyte. B. Scanning electron micrograph of osteoblast on the endosteal surface of a rat long bone. C. Scanning electron micrograph of a human osteoclast cultured for 24 hours on a bone slice (Dempster et al. 2006).

Osteocytes are star shaped cells with long cytoplasmic processes, found in great numbers in compact bone. Osteocytes can be considered as terminally differentiated osteoblasts, which become trapped in the matrix they secrete. Osteocytes make up over 90% of the cells in bone (Dallas and Bonewald 2010). They are highly active cells, and have a high degree of interconnectivity to each other via long cytoplasmic extensions that occupy channels in the bone matrix called canaliculi. Osteocytes occupied a space in the bone called a lacuna. Osteocytes are known to be responsible for detecting and responding to mechanical loading and initiating the bone adaptation process to weight and mechanical challenges (You et al. 2008).

Osteocytes are also involved as regulators of phosphate and calcium homeostasis, by expression of Dentin matrix Protein 1, Phex, Mepe/OF45 (Toyosawa et al. 2001; van Bezooijen et al. 2004). Osteocytes also produce fibroblast growth factor-23 (FGF23) (Feng et al. 2006; Liu et al. 2006a; Liu et al. 2003; Riminucci et al. 2003; Sitara et al. 2004), which is a 32-kDa hormone (Quarles 2008) with a crucial role in phosphate (Pi) homeostasis (Shimada et al. 2001). FGF23 is a member of the subfamily of FGFs that act as hormones/systemic factors due to their ability to interact with FGF receptor (FGFR) in the presence of the Klotho family of proteins (Tsujikawa et al. 2003). FGF23 principally functions as a phosphaturic factor acting as a counterregulatory phosphaturic hormone to maintain phosphate homeostasis in response to vitamin D (Larsson et al. 2004; Liu et al. 2006b; Shimada et al. 2001) and counter-regulatory hormone for 1,25(OH)₂D production (Liu et al. 2006b). An excess of FGF23 causes hypophosphatemia, also suppresses 1,25(OH)₂D (Bai et al. 2004; Larsson et al. 2004; Shimada et al. 2001). Deficiency of FGF23 results in the opposite renal phenotype, with hyperphosphatemia and elevated production of 1,25(OH)₂D (Liu et al. 2006b; Sitara et al. 2004). Supraphysiologic FGF23 levels found in several genetic disorders (such as autosomal-recessive hypophosphatemic rickets and osteoglophonic dysplasia) and in FGF23-producing tumours caused a decreased renal Pi reabsorption and hypophosphatemia resulting in osteomalacia and rickets (White et al. 2006). Parathyroid hormone receptor signalling in osteocytes increases the expression of fibroblast growth factor-23 in vitro and in vivo (Rhee et al. 2011).

Recent *in vitro* studies reported that osteocytes support osteoclast formation and activation when co-cultured with osteoclast precursors (You et al. 2008). Osteocytes are the major source of receptor activator of nuclear factor κ B ligand for osteoclast formation and bone remodelling (Nakashima et al. 2011). It has also been suggested that mechanical loading decreases the osteocyte's potential to induce osteoclast formation by direct cell to cell contact but it is not clear that osteocytes *in vivo* are able to form direct cell to cell contact with osteoclast precursors (You et al. 2008).

Mechanically stimulated osteocytes release soluble factors that can inhibit osteoclastogenesis induced by other cells including bone marrow stromal cells; meaning that osteocytes work as

mechanotransducers by regulating osteoclastogenesis via soluble signals. Negative regulators of the Wnt/ β -catenin pathway such as Dkk1 and sclerostin are highly expressed in osteocytes. Dkk1 is expressed throughout the body but sclerostin is expressed mainly in osteocytes. Mature osteocytes have been shown to produce sclerostin, coded by the gene *SOST* that can inhibit osteoblast activity (Bonewald 2011).

As well as converting mechanical strain into biochemical signals that regulate resorption or formation (Gluhak-Heinrich et al. 2003; Lanyon et al. 1993), osteocytes appear to be involved in the deposit or removal of mineral from lacunae in a process called osteocyte osteolysis (Bonewald 2007; Lane et al. 2006).

Osteocytes have the ability to move within lacunae and extension and retraction of dendrites can occur within canaliculi (Bonewald 2007; Veno et al. 2006).

1.1.3. Osteoblasts

Osteoblasts are responsible for formation and mineralization of bone matrix. Osteoblasts are believed to originate from a common mesenchymal progenitor (Fig. 1. 4) and the differentiation pathway is regulated by expression of the runt-related transcription factor 2 (Runx2)/ core-binding factor α -1 (Cbfa1) and Osterix (OSX) which up-regulate expression of osteoblast-specific genes (Galindo et al. 2005) and activating transcription factor 4 (ATF4) which are known to be crucial for the process (Matsuguchi et al. 2009). Bone morphogenetic proteins (BMP's) such as BMP-7/OP-1 are strongly osteoinductive *in vivo* and *in vitro* (Takahashi et al. 2005), and cause commitment and differentiation of osteoprogenitor cells and pre-osteoblasts into mature osteoblasts.

During differentiation from mesenchymal progenitors various hormones and cytokines regulate osteoblast differentiation. Among these, BMPs are the most potent inducers. The BMPs also induce non-osteogenic cells to differentiate into osteoblast lineage cells. Sonic and Indian hedgehog also play an important role interacting with BMPs to regulate the

differentiation. Cbfa1, a transcription factor from the *runx*-domain gene family, is essential but not sufficient for osteoblast differentiation and bone formation (Yamaguchi et al. 2000).

Osteoblasts have progenitors in common with chondrocytes, myocytes and adipocytes. Primitive osteoprogenitors and related mesenchymal precursors arise in the embryo and some persist into adulthood where they contribute to bone remodelling and fracture healing (Fig. 1. 4).

Investigations of knockout and transgenic mice models of Wnt pathway components including LRP5 and 6, beta catenin, secreted frizzled-related protein 1 and 4, dickkopf-1 and -2, sclerostin, axin-2 and T cell factor-1 have shown that canonical signalling modulates many aspects of osteoblast physiology including proliferation, differentiation, function, mineralization, apoptosis and mechano-sensory perception as well as coupling to osteoclast activity. The Wnts are a large family of signalling molecules (Bodine 2008). These proteins bind to a membrane receptor complex comprised of a frizzled (FZD) G protein coupled receptor and a low density lipoprotein (LDL) receptor related protein (LRP). In recent years canonical Wnt signalling has been reported to play a significant role in the control of bone formation and remodelling. Mutations in LRP5 are associated with low bone mineral density, fractures and osteoporosis-pseudoglioma and “high-bone-mass” syndromes (Ferrari et al. 2004). Also some bone forming agents like bone morphogenic proteins (BMP) and parathyroid hormone have been demonstrated to interact with and utilize components of the Wnt signalling pathway (Bodine 2008).

Additionally, the bioactive phospholipids, lysophosphatidic acid (LPA) has been shown to induce proliferation of primary rat osteoblast through a pathway that involves Gi proteins and cytosolic Ca^{2+} (Grey et al. 2001). Studies in vitro have shown that LPA inhibits apoptosis of primary rat osteoblasts (Grey et al. 2002). It has also been reported that LPA induces osteoblastic differentiation of human mesenchymal stem cells by interacting with the receptors LPA1 and LPA4 (Liu et al. 2010).

The mature osteoblast phenotype appears to be heterogeneous with subpopulations of osteoblasts expressing only subsets of the known osteoblast markers (Aubin 1998a; Aubin 1998b). Within the osteoblast lineage, subpopulations of cells can respond selectively to

physiologic signals, meaning that osteoblast from different bone regions, may exhibit different response to hormonal, mechanical, and developmental stimulation (Lian et al. 2003).

Osteoblasts can also differentiate to become bone lining cells, which form a monolayer on bone surface to seal the bone microenvironment.

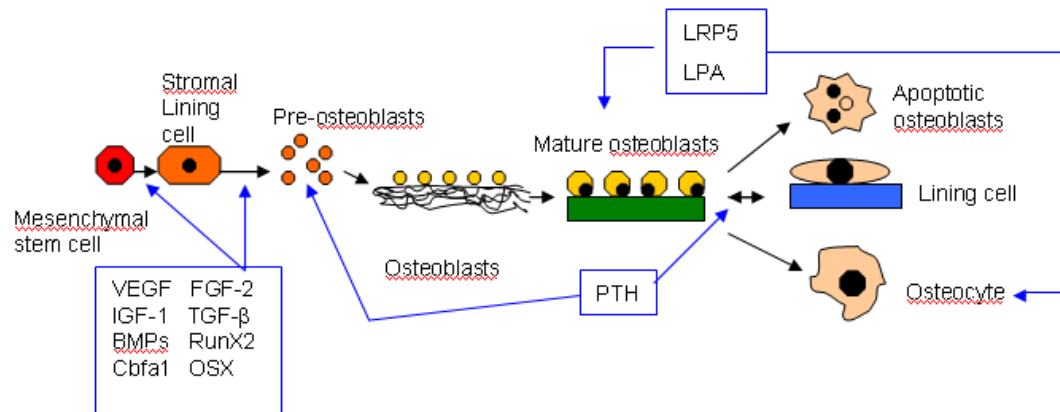


Figure.1.4. Osteoblast differentiation; the blue arrows are indicating the place of action of some of the factors involved in the different stages of differentiation. BMPs (bone morphogenetic proteins), Cbfa1 (core-binding factor α -1), FGF (fibroblast growth factor), IGF (insulin-like growth factor), LPA (lysophosphatidic acid), LRP5 (low density lipoprotein receptor related protein 5), OSX (osterix), PTH (parathyroid hormone), Runx2 (runt-related transcription factor 2), TGF- β (transforming growth factor beta), VEGF (vascular endothelial growth factor).

Mature osteoblasts are mononuclear cells that have a large nucleus, a large Golgi, and extensive endoplasmic reticulum. They secrete collagen and other bone matrix proteins which mineralise to form mature bone. Mineralization is achieved by release of matrix vesicles, which are released by chondrocytes, osteoblasts, odontoblasts, and tenocytes into selected sites where matrix calcification will occur. Additionally the osteoblasts produce alkaline phosphatase which has an important role in the bone mineralization, by degrading pyrophosphate. A substantial amount of bone alkaline phosphatase (ALP) is located at the osteoblast cell membrane.

1.1.4. Osteoclasts

Osteoclasts were discovered by Kolliker in 1873 (Nijweide et al. 1986). These cells are of haematopoietic origin and arise from the monocyte/macrophage cell lineage (Suda et al. 1997; Teitelbaum 2000). Osteoclast differentiation and activity is crucially dependent on the cytokines, receptor activator of NF- κ B ligand (RANKL), MCSF and osteoprotegerin (Dugard et al. 2005). Mature osteoclasts are large multinucleated cells typically with five nuclei and are about 150-200 μ m in diameter. Giant osteoclasts can occur in some diseases, including Paget's disease of bone and bisphosphonate toxicity. These may have dozens of nuclei, and typically express major osteoclast proteins but have significant differences from cells in living bone due to the non-natural substrate (Basle et al. 1988; Weinstein et al. 2009). Osteoclasts are the cells responsible for bone resorption. Bone resorption can be defined as a phase of bone remodelling where the osteoclast precursors are recruited to a new bone resorption site where they differentiate and polarise to form mature osteoclasts and begin to resorb bone (Fig.1.5).

In bone, osteoclasts are found in pits in the bone surface which are called resorption bays, or Howship's lacunae. Osteoclasts are characterized by a cytoplasm with a homogeneous, "foamy" appearance, due to a high number of vesicles and vacuoles. These vacuoles include lysosomes filled with acid phosphatase. Osteoclast rough endoplasmic reticulum is sparse, and the Golgi complex is extensive (Holtrop and King 1977; Vaananen et al. 2000).

At a site of active bone resorption, the osteoclast forms a specialized cell membrane, the "ruffled border," that opposes the surface of the bone tissue. This extensively folded or ruffled border facilitates bone removal by dramatically increasing the cell surface for secretion. Once activated, osteoclasts move to areas of micro-fracture in the bone by chemotaxis. The sealing zone is the attachment of the osteoclast's plasma membrane to the underlying bone. Sealing zones are bounded by adhesion structures called podosomes. Attachment to the bone matrix is facilitated by integrin receptors, such as α v β 3, via the specific amino acid motif Arg-Gly-Asp in bone matrix proteins. The osteoclast releases hydrogen ions through the action of carbonic anhydrase ($\text{H}_2\text{O} + \text{CO}_2 \rightarrow \text{HCO}_3^- + \text{H}^+$) through the ruffled border into the resorptive cavity, acidifying and aiding dissolution of the mineralized bone matrix into Ca^{2+} , H_3PO_4 , H_2CO_3 , water and other substances. Additionally several hydrolytic enzymes, such as members of the cathepsin and matrix metalloprotease (MMP) groups, are released to digest the organic components of the matrix. Upon polarization of the osteoclast over the site of resorption,

cathepsin K is secreted from the **ruffled border** into the **resorptive pit**. Cathepsin K, along with reactive oxygen species generated by TRAP, further degrades the bone extracellular matrix.

Several other cathepsins are expressed in osteoclasts including cathepsins B, C, D, E, G, and L. The function of these cysteine and aspartic proteases is generally unknown within bone, and they are expressed at much lower levels than cathepsin K (Vaananen et al. 2000).

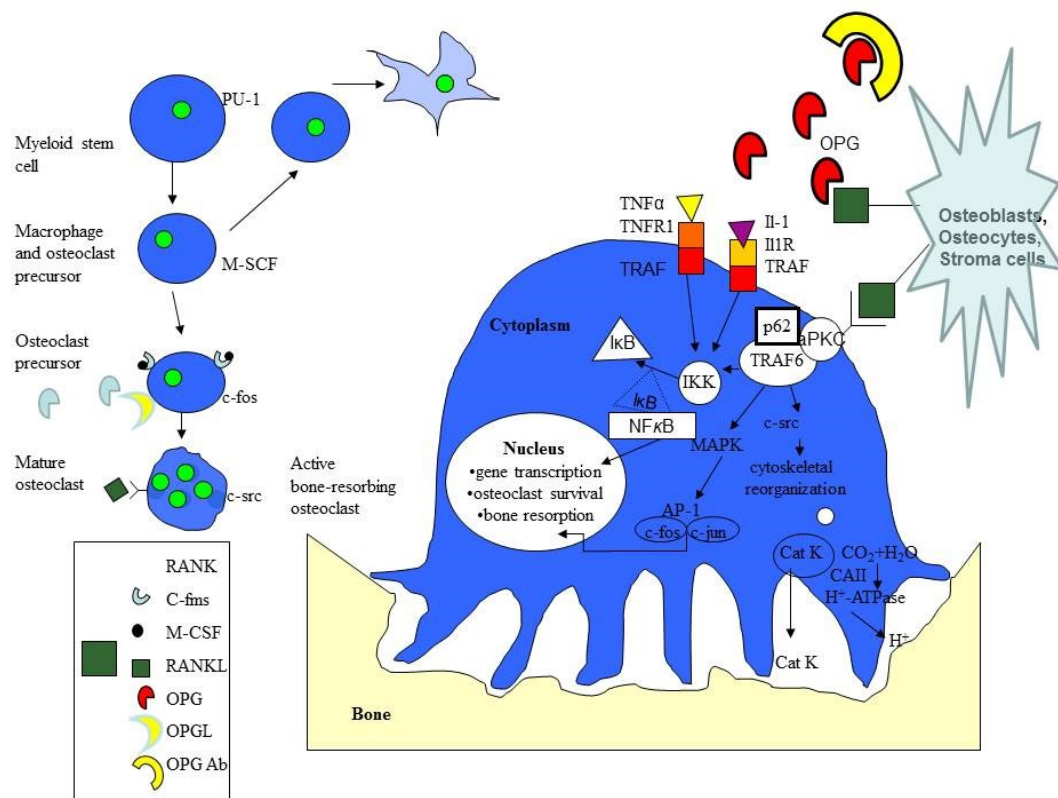


Figure.1.5. Osteoclast Differentiation and factors involved in osteoclastogenesis. Osteoclasts differentiate from a myeloid stem cell; PU-1 is required for determination of myeloid stem cells to the monocyte-macrophage lineage. M-CSF is required for proliferation and survival of cells in the monocyte-macrophage lineage. Osteoblasts/stromal cells are essentially involved in the activation as well as differentiation of osteoclasts through a mechanism involving cell-to-cell contact between osteoblasts/stromal cells and osteoclast precursors/osteoclasts. Osteoclast differentiation factor (ODF, also called RANKL/OPGL/TRANCE) and M-CSF are two essential factors produced by osteoblasts/stromal cells for osteoclastogenesis. c-fos is required for differentiation to osteoclast precursor. c-src required for polarization to allow bone attachment. RANKL is required for fusion and activation. Cathepsin K, carbonic anhydrase II, H⁺ATPase, Tartrate-resistant acid phosphatase and α_vβ₃ integrin/vitronectin receptor are all important for the bone-resorbing activity of osteoclasts.

1.1.5. RANKL

RANKL is known as a type II membrane protein and is a member of the tumor necrosis factor (TNF) superfamily, is an essential cytokine for osteoclastogenesis (Boyle et al. 2003; Suda et al. 1995) a protein encoded by TNFSF11; RANKL can be membrane-bound on osteoblasts and activated T cells or secreted by other cell types (Cenci et al. 2000; Suda et al. 1999; Theill et al. 2002). It has been recently reported that purified osteocytes express RANKL at higher levels than osteoblasts or bone marrow stromal cells (Nakashima et al. 2011). This raises the possibility that RANKL secreted by osteocytes may also regulate bone resorption (Xiong et al. 2011).

RANKL exists in two forms, a soluble form (sRANKL) and a membrane bound form thought to be responsible for initiating osteoclast formation (Boyle et al. 2003; Theoleyre et al. 2004; Xiong et al. 2011). RANKL interacts directly with its cognate receptor, RANK, on the surface of cells in the osteoclast lineage (Suda et al. 2002).

RANK does not possess innate kinase activity to mediate downstream signalling. Like other members of the TNF superfamily it recruits TNF receptor –associated factors (TRAFs), which bind other proteins that play a role in signal transduction.

RANKL is necessary and sufficient, in the presence of small amounts of M-CSF, for the differentiation, maturation and activation of osteoclast from precursors in the osteoclast/macrophage lineage (Lacey et al. 1998; Quinn et al. 1998; Tsukii et al. 1998). Together with DC-STAMP, RANKL is responsible for promoting fusion of mononuclear committed osteoclast precursors, to form mature osteoclasts. Furthermore, RANKL promotes activation of osteoclast by enhancing polarization, ruffled border formation, and by preventing apoptosis (Boyce et al. 2003). RANKL knockout mice develop severe osteopetrosis which is due to a total absence of osteoclasts (Kong et al. 1999). Osteopetrosis, also known as marble bone disease and Albers-Schönberg disease, is an extremely rare inherited disorder whereby the bones harden, becoming denser, can cause bones to dissolve and break. It can cause

osteosclerosis (Lam et al. 2007). The cause of the disease is understood to be malfunctioning osteoclasts (Coudert et al. 2015); in osteopetrosis, the number of osteoclasts may be reduced, normal, or increased. Most importantly, osteoclast dysfunction mediates the pathogenesis of this disease (Askmyr et al. 2008). The disease is caused by underlying mutations that interfere with the acidification of the osteoclast resorption pit (Sobacchi et al. 2013).

RANKL is also expressed in lymph nodes, thymus, mammary glands and lung and at low levels in a variety of other tissues including spleen and bone marrow (Kearns et al. 2008). It is expressed by synovial cells and activated T cells in joints of patients with inflammatory arthritis and contributes at least in part, to the joint destruction seen in patients with rheumatoid arthritis. RANKL also stimulates the release of osteoclast precursor into circulation and apparently osteoclast themselves regulate the egression of hematopoietic stem cells from niches within the marrow under the control of RANKL (Kollet et al. 2006). Thus, RANKL-induced osteoclast activation appears to regulate haematopoietic stem cell mobilization as part of homeostasis and host defence mechanism linking bone remodelling with the regulation of haematopoiesis (Boyce and Xing 2008b). RANKL is also expressed in epithelial cells in mammary gland lobules during pregnancy and is required for hyperplasia of this cell during lactation and milk production in mice (Fata et al. 2000).

T cell production of RANKL also induces expression of IFN β by activated osteoclasts which acts through c-Fos to negatively regulate osteoclast formation; IFN β inhibits the differentiation by interfering with the RANKL—induced expression of c-Fos (Takayanagi et al. 2002b). This may be enhanced by T cell produced IFN γ which degrades TRAF6, an essential adapter protein for RANK signalling (Takayanagi et al. 2002a). IFN- γ has been reported to be a strong suppressor of osteoclastogenesis in vitro through inhibition of RANKL signalling (Takayanagi et al. 2000); however, its effects on osteoclast formation are controversial.

1.1 6. RANK

RANK is a member of the TNF receptor superfamily, protein encoded in humans by TNFRSF11 gene. It is widely expressed at mRNA level by osteoclast-like cells and mononuclear cells in contact with the cortical and trabecular bones surface (Ikeda et al. 2001). It is also expressed by skeletal muscle, thymus, liver, colon, small intestine, adrenal glands and by several human cell lines (Anderson et al. 1997). It appears to be expressed in fewer tissues than RANKL at the protein level. However, in addition to osteoclast precursors, RANK is expressed by mature osteoclasts, pre-osteoclasts, dendritic cells, breast and prostate cancers cells (Kim et al. 2006), CD14⁺ (monocytoid), CD19⁺ (B-lymphoid), CD56⁺ (NK cell), giant cell tumour of bone (GCT), and glycophorin A⁺ erythroid progenitors. Minor populations of T lymphocytes and bone marrow hematopoietic progenitors also expressed cell surface RANK (Atkins et al. 2006).

In 2000 activating mutations (activating mutations refers to a substitution of one amino acid residue by another that confers a new higher activity upon the protein) in exon 1 of RANK have been reported in humans to account for the increased osteoclast formation, activity and osteolysis seen in some patients with familial Paget's disease and familial expansile osteolysis (FEO) again underlying the importance of the system for osteoclast regulation in humans (Hughes et al. 2000). Familial Paget's disease is the cases of Paget's with a positive family history; the line is blurry to define Sporadic cases, which literally mean scattered or isolated, but mainly sporadic Paget's disease are the cases where it is not family history of the disease. In 2004 a deletion mutation was reported that causes a lethal autosomal recessive osteopetrosis, confirming the importance of RANK for osteoclastogenesis (Kapur et al. 2004).

Targeted deletion of RANK causes osteopetrosis in mice. RANK^{-/-} mice has increased numbers of CD11b⁺ osteoclast precursors in their spleen and peripheral blood, meaning that the defect is caused by failure of differentiation rather than a defect on survival of precursors. In RANK^{-/-} mice the teeth fail to erupt and the growth is reduced in comparison with unaffected littermates, craniofacial defects also occur (Kapur et al. 2004).

1.1. 7. Osteoprotegerin

Osteoprotegerin (OPG) is a decoy receptor for RANKL and a member of the TNFR superfamily. It is a protein encoded by the tumour necrosis factor receptor superfamily member 11B (TNFRSF11B) gene (Hofbauer et al. 2001).

Osteoprotegerin is homologous to RANK and acts as a decoy receptor for RANKL. By binding RANKL, OPG prevent the RANKL-RANK interaction and signalling inhibiting of osteoclast formation, differentiation and survival (Hofbauer et al. 2001).

OPG is secreted by multiple cells and tissues, including osteoblasts, B cells, heart, kidney, liver, and spleen. It is mainly produced by B cells in bone marrow in normal mice (Boyce and Xing 2008a) . Osteoclast numbers and activity may increase if there is a change in the RANKL/OPG ratio due to either an increase, decrease or a change in both that leads to a change in the ratio in favour of RANKL.

The osteo-protective role of OPG in humans has been confirmed by the report of homozygous partial deletions of *TNFRSF11B* in patients with juvenile Paget's disease, an autosomal recessive disorder in which affected individuals have increased bone remodelling, osteopenia, bone expansion, deformity and fractures (Whyte et al. 2002). The expression of OPG is regulated in osteoblasts by a variety of cytokines, hormones, growth factors (Theoleyre et al. 2004), and Wnt/ β -catenin (Glass et al. 2005). Additionally, autoantibodies against osteoprotegerin have been described in a male patient with celiac disease who presented with severe osteoporosis and high bone turnover (Riches et al. 2009). The antibodies are able to block the inhibitory effect of the osteoprotegerin on signalling by the receptor activator of nuclear factor (NF)- κ B (RANK); also the autoantibodies have been described in some additional cases with severe osteoporosis and patients with celiac disease (Real et al. 2015; Riches et al. 2009)

1.1.8. M-CSF

Macrophage Colony-Stimulating Factor (M-CSF) is a cytokine required for the survival and proliferation of osteoclast and macrophage precursors until their complete maturation (Fig. 1. 4). The active form of M-CSF is found extracellularly as a disulphide-linked homodimer, and is thought to be produced by proteolytic cleavage of a membrane-bound precursor. M-CSF has been shown to be essential for differentiation of osteoclast precursors in mice and humans *in vitro* and *in vivo* (Fujikawa et al. 1996; Sarma and Flanagan 1996; Suda et al. 1995; Tanaka et al. 1993).

Reflecting the fact that M-CSF is essential for differentiation, mice with a point mutation in the M-CSF gene (op/op mice) have severe osteopetrosis due to absence of osteoclasts (Yoshida et al. 1990).

Macrophage colony-stimulating factor is produced by many cell types, including fibroblast, bone-marrow stromal cells, osteoblasts, and activated monocytes/macrophages. M-CSF expression by osteoblasts and stromal cells is required for progenitor cells to differentiate into osteoclasts in response to RANKL, but M-CSF alone is not sufficient to support osteoclast differentiation.

1.1.9. DC-STAMP

Dendritic Cell-specific Trans membrane protein (DC-STAMP) is a member of the trans membrane 7 superfamily of receptors and is encoded by the TM7SF4 gene. The protein is expressed specifically by antigen presenting dendritic cells and osteoclast precursors. Human and murine DC-STAMP proteins are highly conserved and localized to the endoplasmic reticulum of immature dendritic cells and translocate towards the Golgi compartment upon maturation

(Eleveld-Trancikova et al. 2005; Sanecka et al. 2011).

The expression of DC-STAMP is induced in osteoclast precursor cells by RANKL and other osteoclastogenic cytokines.

Inhibition of DC-STAMP produces defects in osteoclast multinucleation, suppresses the formation of multinucleated osteoclast-like cells and reduces bone-resorbing activity causing osteopetrosis. Osteoclast fusion and multinucleation is controlled by DC-STAMP. Experiments in knock-out mice lacking expression of DC-STAMP, confirmed the function of DC-STAMP, as a critical regulator of the fusion of osteoclast precursors (Yagi et al. 2005).

Additionally the lack of DC-STAMP also affects foreign body giant cell formation by macrophage cell fusion. The immunological and biological processes where DC-STAMP is involved in, are only recently emerging. Some data have suggested involvement of DC-STAMP in phagocytosis and antigen presentation, which has been demonstrated by the fact that aged DC-STAMP knock-out mice show symptoms of autoimmune diseases (Hwang et al. 2014; Sanecka et al. 2011; Sawatani et al. 2008).

1.1.11. Parathyroid hormone, Ca^{2+} and Calcitriol

Parathyroid hormone (PTH) is an 84 amino acid peptide secreted by parathyroid glands. PTH is one of the two major hormones modulating calcium and phosphate homeostasis, the other being calcitriol (Potts 2013). The major regulatory signal for PTH secretion is serum calcium; which inversely affects PTH secretion. High levels of PTH, as seen in primary and secondary hyperparathyroidism, increase osteoclastic bone resorption. Low levels of PTH, delivered in an intermittent manner, seem to increase osteoblastic bone formation. PTH paradoxically can stimulate resorption when administered in a continuous fashion, or bone formation when administered intermittently (Kroll 2000). Bone resorption can be triggered by PTH in response to hypocalcemia; 99% of the body's Ca^{2+} is present in bones.

Low plasma calcium as already mentioned, stimulates PTH release, and PTH acts to resorb Ca^{2+} from the pool in bone and to enhance renal re-absorption of Ca^{2+} . A high level of calcium in plasma, stimulates calcitonin secretion which lowers plasma calcium by inhibiting bone resorption. Ca^{2+} is highly involved in mechanical loading signal transduction which is also called mechanotransduction. The presence of voltage operated calcium channels (VOCCs) in

bone cells is critical for enabling intracellular calcium fluxes to occur in osteoblast in response to loading (El-Haj et al. 1990). Patch clamping measurements have revealed the presence of several types of calcium channels, both voltage-sensitive and voltage-independent. These calcium channels, often coupled with intracellular calcium release, can respond to hormonal, cytokine and especially mechanical stimulation (Duncan et al. 1998). In addition, it has been shown that the production of matrix proteins, such as osteopontin and osteocalcin, are elevated in response to mechanical loading and that this response is strongly inhibited by the calcium channel blocker, nifedipine, and enhanced by the calcium channel agonist Bay K8644 (Walker et al. 2000).

Calcitriol, also called 1, 25-dihydroxycholecalciferol or 1, 25-dihydroxyvitamin D₃, is the hormonally active form of vitamin D. Calcitriol increases the level of calcium in the blood by increasing the intestinal calcium absorption, specifically by increasing the calcium uptake through the brush border membrane of the enterocyte. Vitamin D also regulates skeletal metabolism through the RANK pathway. Additionally vitamin D promotes the mineralization of osteoid and stimulates bone resorption by mature osteoclast, indirectly by promoting cell recruitment and interaction with osteoblast and the fusion of monocytic precursors to osteoclast. Vitamin D also regulates the expression of several bone proteins, as osteocalcin. It promotes the transcription of osteocalcin and has bidirectional effects on type I collagen and alkaline phosphatase gene transcription (Adams et al. 1989; Zaloga et al. 1984).

1.1.12. Other factors involved in bone resorption

Many other factors play a role in the regulation of osteoclast differentiation and function. There are two critical steps in the generation of mature osteoclast: step one is the commitment of the progenitor cells to osteoclast precursor cells, which involves the activation of osteoclast marker genes such as **Tartrate-resistant acid phosphatase (TRAcP)** and the calcitonin receptor; Step two, which is the fusion of TRAcP-positive mononuclear cells to form multinucleated osteoclasts. Pathological disruption of osteoclastic bone resorption leads to a condition called osteopetrosis, which is characterised by a marked increase in bone density

(Balemans et al. 2005). Analysis of mice with mutations that result in the osteopetrotic phenotype have provided insights into the important factors involved in the differentiation of myeloid stem cells into active osteoclasts (Fig. 1. 5).

Src tyrosine kinase plays an essential role, because its expression is required for the cytoskeletal protein rearrangement that results in formation of the ruffled border, which attaches the cell tightly to the bone surface (Boyce et al. 1992; Boyce et al. 1993). Within this sealing zone, hydrogen and chloride ions are secreted through proton and chloride pumps and combine to form hydrochloric acid, which dissolves the mineral in the bone matrix. Src or other Src family members also appear to mediate RANKL-mediated osteoclast survival in a signalling pathway that involves phosphorylation of PI3 kinase, Akt, BAD and caspase 9, where caspase 9 prevents activation of the apoptosis pathway (Wong et al. 1999; Xing et al. 2001).

The transcription factor PU-1 is essential for differentiation cells to cells of the osteoclast/macrophage lineage and PU.1 knockout mice are not only devoid of osteoclasts leading to osteopetrosis, but also lack macrophages within the marrow, lungs and liver (Tondravi et al. 1997). **C-fos** is also an essential transcription factor in osteoclast differentiation and mice lacking c-fos develop severe osteopetrosis (Wang et al. 1992)

Cathepsin K is another essential protein involved in bone remodelling and resorption. This protein, which is member of the peptidase C1 protein family, is predominantly expressed by Osteoclasts. Osteoclasts synthesize and secrete cathepsin K into the space between the osteoclast and the bone surface, where it degrades bone collagen. RANKL signalling via the **calcineurin-calcium-NFAT signalling cascade** plays an important role in the regulation of cathepsin K expression (Troen 2006).

The nuclear factor of activated T-cells (NFAT) is a family of transcription factors where NFATc1, in particular, is crucial for multiple seemingly unrelated biologic processes, including heart valve formation (Wu et al. 2011), T-cell activation, osteoclast development and the mitigation of hair follicle stem cell proliferation (Aliprantis and Glimcher 2010). **NFAT** controls bone resorption in vivo by stimulating the differentiation and functioning of

osteoclast but not their survival (Ikeda et al. 2006); NFAT, Mitf and various components of AP-1 enhance osteoclast formation and bone resorption, while IFN-gamma, calcitonin, estradiol, and calcium have an inhibitory effect (Troen 2006).

The fusion of osteoclast precursors to become a multinucleated bone resorbing active cell, require RANKL, DC-STAMP (Yagi et al. 2005) as mention before.

Bone resorption relies on the extracellular acidification function of vacuolar (V-) ATPase proton pump(s) present in the plasma membrane of osteoclasts. **Atp6v0d2**, is an essential component of the osteoclast-specific proton pump that mediates extracellular acidification in bone resorption (Wu et al. 2009), **is a subunit of v-ATPase**, is a component of the V-type H⁺ ATP6i osteoclast proton pump complex that secretes H⁺ from the osteoclast (Lee et al. 2006). Vacuolar ATPases, in the osteoclast plasma membrane, pump protons onto the bone surface which is necessary for bone resorption. They are also responsible for acidifying a variety of intracellular compartments in eukaryotic cells. Vacuolar-type proton transporting ATPase (V-ATPase) is a multi-subunit complex and an enzyme (Finbow and Harrison 1997; Nelson 1992). The ***TCIRG1*** gene through alternate splicing (Smirnova et al. 2005), encodes two proteins with similarity to subunits of the vacuolar ATPase, the encoded proteins seem to have different functions; mutations in the *TCIRG1* locus, cause severe autosomal recessive osteopetrosis (ARO) in humans (Kornak *et al.* 2000). Osteopetrosis is an inherited disorder of impaired bone resorption, where the most commonly affected genes are ***CLCN7*** and ***TCIRG1***, encoding the Cl⁻/H⁺ exchanger *CLC-7* and the $\alpha 3$ subunit of the vacuolar H⁺-ATPase, respectively (Barvencik et al. 2013). Significant association has been reported between ***CLCN7*** polymorphisms with the variance of modulate bone density and bone resorption marker levels in postmenopausal women and with the variability of the autosomal dominant osteopetrosis type II phenotype (Kornak et al. 2006).

As is evident, bone remodelling is a complex process regulated by multiple factors involving a number of cells and their differentiation and in this work I will not claim to mention and describe them all, but we need to underline the fact that while PDB is characterised by increases osteoblast and osteoclast activity, the primary cellular abnormality is generally considered to reside within osteoclast.

1.2. Paget's Disease of Bone

1.2.1. Historical Aspects: Sir James Paget

Paget's disease was classically described by Sir James Paget, who studied medicine at St Bartholomew's Hospital in London in 1834. He was hard working, brilliant and dedicated student.

In 1847 he was accepted as a surgeon at Barts and in 1858 he was Appointed Surgeon Extraordinary to Queen Victoria. In 1874 he received an honorary degree from the University of Cambridge (Paget, Sir James, Bart in Venn, J. & J. A., *Alumni Cantabrigienses*, Cambridge University Press, 10 vols, 1922-1958) at the age of sixty-one he is universally accepted as one eminent men of his profession.

In November 1876 Sir James Paget published a paper regarding a form of chronic bone disease which he called osteitis deformans. This paper described five cases of this disease, which had previously gone largely unrecognized. The main case described, was that of a 46 year old coachman who first visited St Bartholomew's Hospital in 1854 suffering from pain in his lower limbs. James Paget described the disease as "a chronic form of inflammation of the bones". Paget observed that the patient's left shin bone and his left thigh bone were enlarged and deformed. No other abnormality was noted. It was an unknown condition and he was therefore unable to make a diagnosis. In the years that followed the bones of the man's right leg also became enlarged and eventually his legs became bowed to such an extent that he could not bring his knees together. Simultaneously his skull became so large and deformed that he had to buy increasingly larger hats. The patient was so stooped that his head sank onto his chest and his height was reduced by over four inches.

Twenty years after his first consultation the patient developed a rapidly enlarging bone mass in his left forearm which was diagnosed as bone cancer and possibly caused his death two months later. During the post mortem, microscopic examination of the bones showed a

dramatic aberration in the bone remodelling process that normally continues throughout life which Paget thought was inflammatory in nature so he called it osteitis deformans. This is the disease now known as Paget's Disease of Bone.

It was a remarkable feat of observation and diagnostic skill which brought about this diagnosis as another forty years elapsed before the development of X-rays allowing the whole skeleton to be examined in living patients for the first time. It was at this time that doctors realized that PDB was not the rare condition they had thought it to be, and that many older people have one or more bones affected by the disease, even though they may not be experiencing any symptoms. Paget's original description of the condition is so accurate that not much has been added to it since from the clinical point of view, except to mention that PDB is often a hereditary condition.

1.2.2. Paget's Disease of Bone

Paget's Disease of Bone (MIM 167250, 602080) is a focal disorder of the skeleton, characterized by increased and disorganised bone remodelling affecting one or more sites throughout the skeleton. This results in the deposition of abnormal bone which is mechanically weak. It is thought to be primarily a disorder of osteoclasts, although bone formation is also abnormally increased.

The main abnormality is a marked increase in the rate of bone resorption caused by large multinucleated osteoclasts. Radiologically, these areas are seen as an advancing osteolytic wedge in long bones or as osteoporosis circumscripta in the skull. The elevated bone turnover causes a subsequent compensatory increase in bone formation, leading to osteosclerosis and bone expansion (Fig. 1.6). Accelerated deposition of bone in a disorganized fashion, produces a "mosaic" pattern, rather than the normal linear lamellar pattern. The marrow spaces are filled by an excess of fibrous connective tissue with a marked increase in blood vessels, causing the bone to become hypervascular (Mirra et al. 1995a; Mirra et al. 1995b).

PDB it is not a new disease. It has been described in various ancient skeletal remains, one of those being an Anglo-Saxon skeleton dated around 950AD, which was dug up at Jarrow Monastery, and is still available for examination (Wells and Woodhouse 1975). Other archaeological studies demonstrated the occurrence of the disease around 1500AD (Rogers et al. 2002).

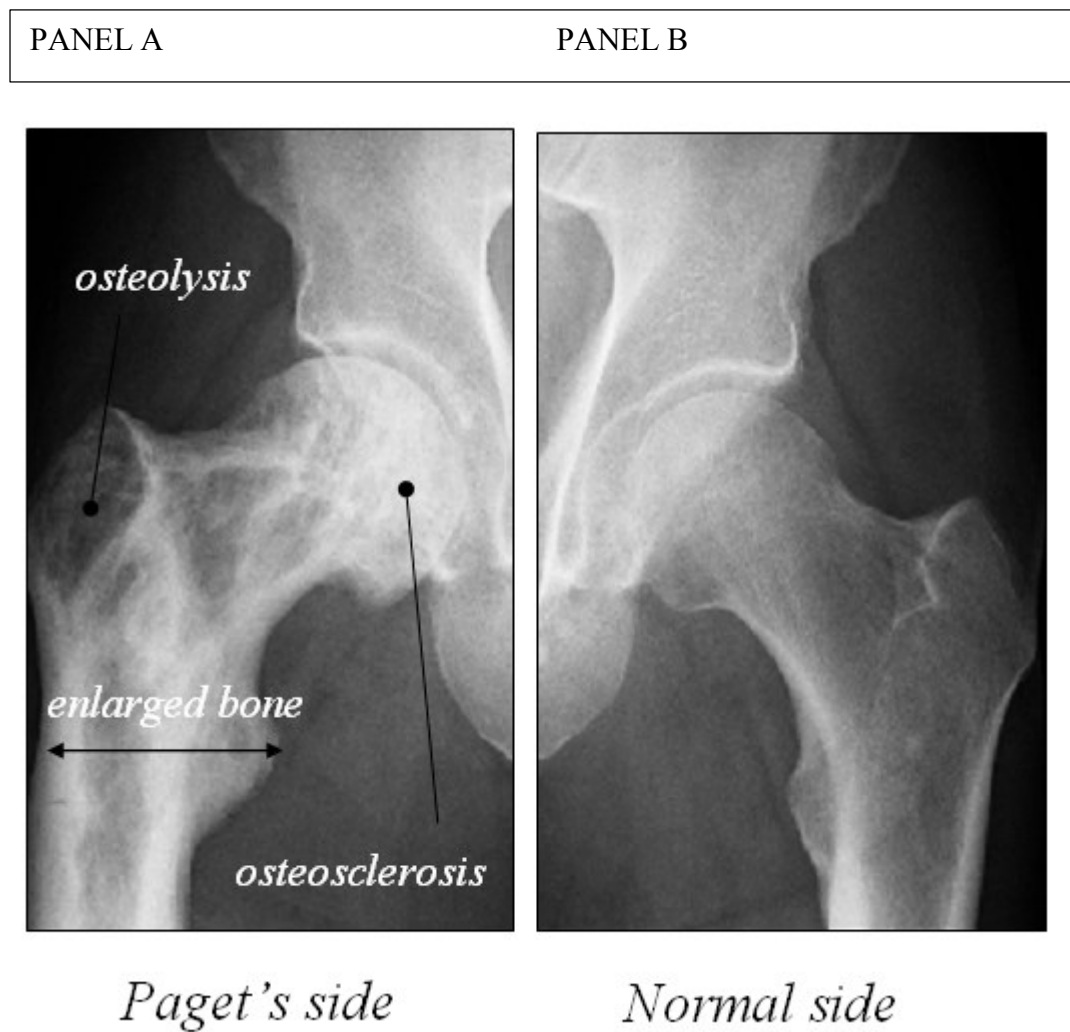


Figure. 1. 6: Panel a; Osteosclerosis, bone expansion and osteolysis caused by elevated bone turnover and the compensatory increase in bone formation. Panel B; compared with the normal side. Radiograph kindly provided by Prof. Stuart Ralston.

The abnormal remodelling processes disrupt normal bone architecture and can lead to the development of several complications including bone pain, deformity, secondary osteoarthritis, nerve compression syndromes and pathological fractures (Visconti et al. 2010). Although sarcomatous degeneration is a rare complication of Paget disease (reportedly less than 0.1%), a high number of osteosarcomas in adults occur in patients with PDB (Merkow and Lane 1990; Moore et al. 1991). Some patients have severe disease which has a major impact on quality of life (Gold et al. 1996; Langston et al. 2007a; van Staa et al. 2002b) but others are completely asymptomatic (van Staa et al. 2002b). The determinants of clinical severity of PDB are poorly understood.

1.2.3. Epidemiology

The prevalence of PDB differs markedly between countries. It is common in the populations of England, France, Italy, and Spain and also in European emigrants to Australia, New Zealand, Netherlands, USA and South Africa. It is rare throughout Asia and in Scandinavia (Barker et al. 1980; Barker et al. 1977; Detheridge et al. 1982; Gardner et al. 1978; Guyer and Chamberlain 1980). Estimates of the prevalence in individual British areas showed that in England and Wales's hospital discharge rates for PDB are higher in the north and west when compared with the south and east. This can even be shown inside Scotland, where the discharge rates for the areas around Glasgow are higher than in the Highlands and Borders (Barker and Gardner 1974).

Paget's disease in New Zealand, England and Wales, is slightly more common in men than in women (Bastin et al. 2009; van Staa et al. 2002b). The disease clearly increases in prevalence with age and more than 90% of patients are older than 40 years of age. The prevalence approaches 10% by 90 years (Gardner et al. 1978).

According to data from US National Health and Nutritional Examination Survey, based on X-ray of the hip, pelvis and spine, the prevalence of PDB has been estimated at 1.3 per 100 women and men age 45-74. In the US it has been estimated that there are over a million affected individual (Altman et al. 2000). On the other hand, many Pagetic patients are asymptomatic and as a consequence they pass undetected by health care professionals, which means that the prevalence of clinically diagnosed cases of PDB is much less. It has been estimated that about 7% of patients with the disorder (apparent on radiographs) reached clinical diagnosis (van Staa et al. 2002b).

There is some evidence that the incidence and severity of Paget's disease is falling (Tiegs et al. 2000) in countries such as Britain and New Zealand (Cooper et al. 1999; Corral-Gudino et al. 2013a; Corral-Gudino et al. 2013b; Doyle et al. 2002; Poor et al. 2006b). In Italy and Spain, however, no secular trend for a decreasing prevalence of PDB has been observed.

1.2.4. Clinical Presentation and Diagnosis

The clinical features of PDB may vary from patients being asymptomatic (involving a single bone) to patients having severe, multiple bone involvement with systemic disease. The most common sites of involvement are pelvis, vertebra and femur. Skeletal complications attributable to PDB included bowing deformities, fractures of affected bones and rarely osteosarcoma. Osteoarthritis is common and about 7% of the cases in the PRISM study required hip or knee replacement (Langston et al. 2007c). Other complications related to PDB included cranial nerve, peripheral nerve and nerve root compression, basilar invagination, hypercalcemia, and congestive heart failure. Also hearing loss may occur (Wermers et al. 2008).

Paget Disease of Bone may present in a wide variety of ways. PDB is often diagnosed incidentally on X-rays obtained for other purposes, in patients who are undergoing biochemical or radiological investigations for other conditions. On the other hand, it is not

unusual for patients to present to the rheumatologist, orthopaedic surgeon or ENT specialist with symptoms specifically related to the disease, such as bone pain, bone deformity, fractures and deafness.

A common manifestation is pathologic fractures, they occur most frequently in the long weight-bearing bones of the lower extremities such as the femoral neck and sub-trochanteric region of the femur and tibia (Merkow and Lane 1990). PDB may also present with pain, increasing deformity, and tenderness over the affected bone. There may be night pain unrelated to change of position. When the spine is affected, neural compression may be the source of presenting problems. When the skull is affected (Fig.1.7), headache and deafness may be observed. Hat size may increase. The cardiovascular system can be involved, due to high output cardiac failure caused by increased blood flow through Pagetic bone.

The diagnosis of PDB is based on the typical features on an X-ray. There may be an increase in serum alkaline phosphatase level, which reflects increase in bone formation, but population based radiologic surveys (Eckhoff et al. 2004b) have shown that about 86% of PDB patients have normal ALP. Hypercalcemia may occur in patients that have been immobilized. Uric acid level may be increased. Hydroxyproline levels are an indicator of bone resorption and lysis, they are found elevated in PDB. Urinary pyridinium/pyridinoline peptides can also be used as markers of bone resorption in PDB. Other biochemical markers such as osteocalcin (OC), C-terminal telopeptide of type I collagen (CTX), deoxypyridinoline (DPD) and procollagen type 1 N-terminal propeptide (P1NP) are found in PDB and can be used to assess bone formation and the response to treatment (Shankar and Hosking 2006). More usually, however, ALP levels are used to assess activity and treatment response in PDB.

Bone turnover markers may provide an assessment of the rate of bone turnover (for bone turnover markers summary see table 1.2 A and B), they are proteins originating from osteoclast and osteoblast activity or fragments released during the formation or degradation of type I collagen. Some of these peptides are sufficiently small to be filtered into urine, while larger fragments may be detected in blood. Bone turnover markers are released during normal bone turnover. The concentrations may rise in metabolic bone diseases (as osteoporosis), other pathological conditions and during physiological processes such as fracture healing and

growth spurts. They cannot be used for screening or diagnosis of specific bone diseases. Their concentrations and patterns may be used by specialist units to monitor treatment response and disease progression in metabolic bone diseases including Paget's disease of bone (Sunethra Devika C Thomas, 2012. www.australianprescriber.com).

Current evidence suggest that bone turnover markers may be useful for monitoring the response to antiresorptive therapy (Bonnick and Shulman 2006; Compston 2009). Intravenous and oral bisphosphonate therapy respectively lead to a decrease in bone resorption markers within days and weeks. The decrease in resorption markers is followed by a decline in bone formation markers. The decrease in the bone turnover markers may be sustained for years after cessation of therapy in patients who have been treated for several years.

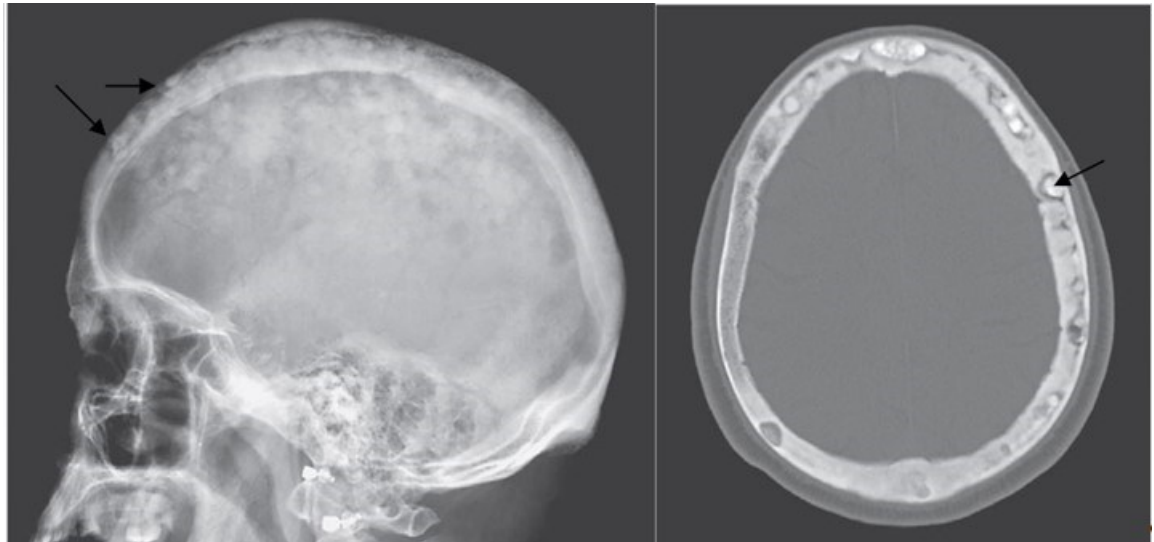
However a significant change in bone turnover markers after starting therapy confirms compliance(Sunethra Devika C Thomas, 2012. www.australianprescriber.com).

Marker	Abbr viation	Method	Specimen
Bone-specific alkaline phosphatase	BAP, bone ALP	IRMA, EIA, Electrophoresis, precipitation.	Serum
Osteocalcin	OC	RIA, IRMA ELISA.	Serum
C-terminal propeptide of type I procollagen	PICP	RIA, ELISA	Serum
N-terminal propeptide of type I procollagen	PINP	RIA, ELISA	Serum

Table. 1.2. A: Markers of bone turnover: Bone formation.

Marker	Abre viation	Method	Specimen
Hydroxyproline, total and dialysable	Hyp	HPLC, Colorimet ry	Urine
Hydroxylysine-glycosides		HPLC, ELISA	Urine (serum)
Pyridinoline	PYD	HPLC, ELISA	Urine, Serum
Deoxypyridinoline	DPD	HPLC, ELISA	Urine, Serum
Carboxyterminal cross-linked telopeptide of type I collagen	ICTP, CTX-MMP	RIA	Serum
Carboxyterminal cross-linked telopeptide of type I collagen	CTX-I	ELISA, RIA	Urine (a- /β) Serum (β only)
Aminoterminal cross-linked telopeptide of type I collagen	NTX-I	ELISA, CLIA, RIA	Urine, Serum
Collagen I alpha 1 helicoidal peptide	HELP	ELISA	Urine
Bone Sialoprotein	BSP	RIA, ELISA	Serum
Osteocalcin fragments	ufOC, U-Mid- OC,U- LongOC	ELISA	Urine
Tartrate-resistant acid phosphatase	TRAcP	Colorimet ry, RIA, ELISA	Plasma, Serum
Cathepsins		ELISA	Plasma, Serum

Table. 1.2. B: Markers of bone turnover: Bone resorption.



PANEL A

PANEL B

Figure.1. 7: A 63 year's old man with a long history of sinusitis and 3 weeks of frontal headache. The physical examination was ordinary. The alkaline phosphatase level was elevated at 434 IU per litre (upper limit of the normal range is 129). The serum calcium level was within normal limits. Radiography of the skull (Panel A) showed thickening of the outer and inner tables of the cranial bones, widening of the diploe, and a "cotton wool" appearance caused by irregular areas of sclerosis (arrows). Computed tomography of the skull (Panel B) confirmed bony expansion, cortical bone thickening, and irregular areas of sclerosis (arrows). These imaging findings reflect the mixed osteolytic and osteoblastic phases of PDB, resulting in accelerated bone turnover with bone deposition and expansion. The patient was treated with alendronate, which resulted in improvement in frontal headache (Bhargava and Maki 2010).

Radionuclide bone scans are more sensitive than plain X-rays in detecting PDB. Bone scans are used to screen for PDB and evaluate skeletal involvement of the disease. The bone scan appearance in PDB is usually pathognomic, but is recommended to confirm the diagnosis by conventional radiography of at least one site (Selby et al. 2002).

1.2.5. Treatment

In the case of PDB, a cure is not available. Treatment is intended to reduce or stabilize the symptoms, suppress bone turnover, reduce pain and improve the patient's quality of life.

The general recommendation is that medication should be given when the patient is experiencing bone pain which appears to be directly associated with the disease. It has also been suggested that prophylactic treatment should be considered for patients who will require surgery, those with active symptoms in skull, long bones or vertebra involvement; also for those patients with disease located in bones in close proximity to major joints, with a risk of developing osteoarthritis and in patients with several bones affected. There is no evidence this is beneficial however.

Many antiresorptive therapeutic agents have been used over the years for the treatment of PDB including mithramycin (plicamycin), glucagon, actinomycin D, calcitonin (porcine, salmon, or human) and gallium nitrate. Most of them have been replaced by bisphosphonates which are the current treatment of choice. The aim of these therapies is to improve symptoms controlling the metabolic activity of the disease (Merkow and Lane 1990).

Calcitonin was the first osteoclast inhibitor to be used in the treatment of PDB. It was proven to be effective in suppressing bone turnover and improving bone pain in PDB (Martin 1980). Comparing with bisphosphonates, calcitonin is more expensive, generally less effective in suppressing bone turnover and has shorter duration of action. Additionally, some patients develop side-effects such as nausea, vomiting and flushing. Resistance may also occur with continued use due perhaps to down-regulation of calcitonin receptors on the osteoclast or development of antibodies (Dube et al. 1973).

Bisphosphonates are drugs that inhibit osteoclast. They are called bisphosphonates because they have two phosphonates groups and are similar in structure to pyrophosphate.

Bisphosphonates were developed in the 19th century as water softening agents, but were first investigated in the 1960's for use in disorders of bone metabolism.

Bisphosphonates inhibit osteoclast activation and bone resorption by causing apoptosis, or cell death, thus slowing bone loss (Weinstein et al. 2009).

Bisphosphonates work according to one of two main mechanisms of action, depending on the chemical nature of the side chain attached to the bisphosphonate core (fig.1.8). Non-nitrogen bisphosphonates (such as etidronate, clodronate, and tiludronate) (Fig. 1.9) inhibit bone resorption by generating a toxic analog of adenosine triphosphate, targeting the mitochondria (Rogers 2003). The second mechanism of action occurs with the nitrogen-containing bisphosphonates (as alendronate, ibandronate, pamidronate, risendronate and zoledronic acid); they are more potent, and act by targeting pyrophosphatase synthase enzyme in the mevalonate pathway (Rogers 2003).

Bisphosphonates: Basic structure and structure of Zoledronate

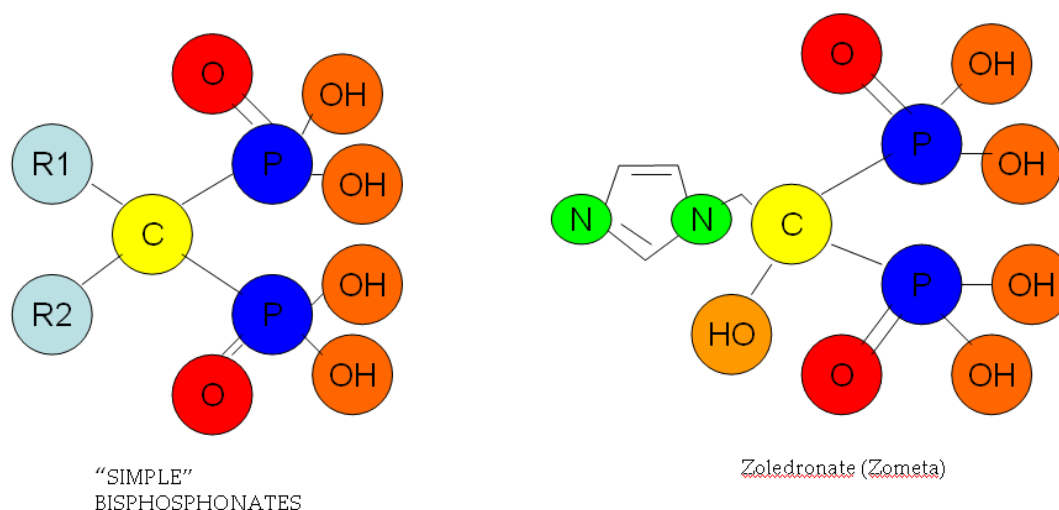
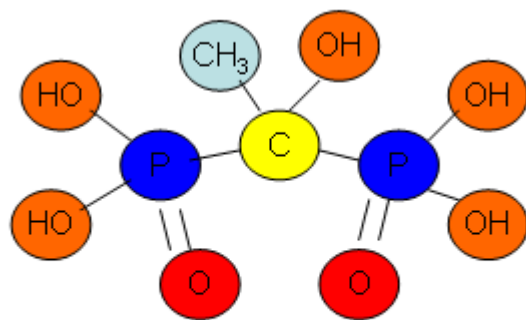


Figure.1.8. The figure shows at the left side, “simple Bisphosphonate” with two additional chains (R1 and R2). R1 structure together with the P-C-P are primarily responsible for binding to bone mineral and for the physicochemical action of the molecule, an hydroxyl group in this position, provides optimal condition for this function. R2 is believed to be responsible for the antiresorptive action, small modifications in this group, translate into marked differences in antiresorptive potency. The presence of a nitrogen molecule in an alkyl chain or in a ring structure, in R2 greatly enhances the antiresorptive potency and specificity of the bisphosphonate. As in the right side we have a molecule of zoledronate, one of the newer potent bisphosphonates containing nitrogen in the R2 chain and a hydroxyl group on R1 (Michou and Brown 2011; Papapoulos 1997; Russell 2007).

As well as being used in PDB, bisphosphonates are used in the prevention and treatment of osteoporosis, bone metastase (with or without hypercalcemia), multiple myeloma, primary hyperparathyroidism, osteogenesis imperfect and other conditions characterised by raised bone turnover.

Additionally, radio-labelled bisphosphonates are used in scintigraphy to demonstrate disease distribution and activity of PDB, since these compounds are taken up by bone in proportion to local turnover (Reid and Hosking 2011).



Etidronic acid (trade name *Didronel*)

Figure.1.9: Etidronic acid. Didronel (the bisphosphonate used for treatment) contains etidronate disodium, the disodium salt of (1-hydroxyethylidene) diphosphonic acid for oral administration, basically is sodium added in one of the OH group for each P.

While several potent bisphosphonates have been licensed for the treatment of PDB (Miller et al. 1999; Reid et al. 2005; Siris 1996) clinical trials of these agents have mainly been focused on evaluating the short term responses of biochemical markers as ALP (Altman et al. 1973; Langston et al. 2007b; Miller et al. 1999; Recker et al. 2008; Reid et al. 1996; Reid et al. 2005). Therefore, in most of these studies, ALP has been the primary end-point. While ALP is a marker of metabolic activity in PDB, it has not been shown that patients with lower levels of ALP have a more favourable clinical outcome (Langston et al. 2007b; Langston et al. 2007c). Indeed, the PRISM study showed that intensive bisphosphonate therapy conferred no clinical advantage over symptom-driven management in patients with established PDB. It was also shown that either management strategy had a significant beneficial impact on pain, quality of life, fractures, hearing loss, adverse events or serious adverse events (Langston et al. 2007c).

Zoledronic acid (marketed by Novartis under the trade names Zometa, Zomera, Aclasta and Reclast) is the most potent licensed bisphosphonate. It has been shown to be effective in the treatment of osteoporosis and in the prevention of skeletal related events in malignancy (Black et al. 2007; Lyles et al. 2007; Reid et al. 2002). It is also used to prevent recurring fractures in patients with previous hip fracture (Craig et al. 2011; Zidrou et al. 2010). It is given as a single dose of 5mg intravenously (Michou and Brown 2011; Reid et al. 2005). Comparative trials have been published evaluating the relative efficacy of the bisphosphonates in the treatment of PDB with differing protocols. These trials still showed that zoledronic acid 5 mg could give an extended biochemical remission in 98% of the patients with one single dose, compared with 57% for risedronate (Michou and Brown 2011; Reid et al. 2005; Reid and Hosking 2011). Resistance has not been observed so far (Papapoulos et al. 2006), which is common with etidronate and pamidronate. Also normalization of serum total alkaline phosphatase was achieved at six months in 93% (Martini et al. 2007; Merlotti et al. 2007). A post infusion syndrome (a flu-like illness) occurring in about 15% of patients treated (mostly at the first infusion) is the main adverse effect (Reid et al. 2005; Reid and Hosking 2011).

Zoledronic acid has the highest affinity for hydroxyapatite *in vitro* and is the most potent inhibitor of the bisphosphonate target enzyme farnesyl diphosphate synthase, also known as farnesyl pyrophosphate (FPPS) (Dunford et al. 2001; Nancollas et al. 2006). Also zoledronic acid produces sustained response, allowing arrest of bone turnover for many years in most patients with only a single infusion (Reid et al. 2011). After 5-6 years the therapeutic response is still maintained in 87% and 38%, respectively, with improvement in quality of life scores in the zoledronate-treated patients (Reid et al. 2011).

1.2.6. Role of Genetics in Paget's Disease of Bone

In 1876 when Sir James Paget first described the disease, he did not note a genetic predisposition since his index case had no other family members affected.

Subsequently there have been many reports of familial aggregation in PDB. In 1883 Pick made the first report of the occurrence of Paget's disease in more than one member of a family. He described a father-daughter pair with Paget's disease (Pick 1883). After that, Lunn reported in 1885 a case where two sisters had PDB (Lunn J.R. 1885).

In 1947 Boyd suggested that PDB may be familial, and added that he knew of two families in each of which three cases occurred (Boyd 1947; Montagu 1949). Additionally Montagu suggested that: "the predisposition to Paget's disease, when inherited, is transmitted as an incompletely dominant gene carried on a X-chromosome" (Boyd 1947; Montagu 1949). We know now this was incorrect and his report was consistent with autosomal dominant transmission. In 1983 Sofaer *et al* reported that Paget's disease shows a degree of familial aggregation (Sofaer et al. 1983). Siris reported that risk in siblings of cases was higher when a parent was affected ($22.1 \pm 8\%$ SEM) compared with the situation when both parents were unaffected ($6.7 \pm 1.1\%$ SEM) (Siris et al. 1991). Migrant studies have shown that the risk of the disease remains high in subjects who move from high-prevalence regions to low-prevalence regions, supporting the influence of a genetic component to the pathogenesis (Gardner et al. 1978). It has been estimated that between 15-40% of patients have a positive family history of the disease (Morales-Piga et al. 1995; Siris et al. 1991; Sofaer et al. 1983), these cases are described as familial cases of PDB.

Furthermore, the risk of developing PDB is 7-10 times higher in relatives of PDB patients compared with controls (Siris et al. 1991; Sofaer et al. 1983). Patients with PDB, with a positive family history are more likely to have a severe disease, with an earlier age at

presentation, polyostotic involvement and bone deformity, compared with those patients without family history (Morales-Piga et al. 1995; Siris et al. 1991).

We can defined “familial Paget’s disease of bone” as the disease inherited in an autosomal dominant manner, with incomplete penetrance, 15% to 20% of Pagetic individuals have a first-degree relative with the disease (Seton et al. 2003a; Siris et al. 1991; Sofaer et al. 1983). Several families have been described in which Paget’s disease affects several generations. The cases with family history tent to have an earlier age of onset, a trend to a higher incidence of bone deformity was observed and an increased fracture rate (Seton et al. 2003b). Many individuals do not have a positive family history of the disease (Hocking et al. 2000) or have not being discovered, in this case they are called “sporadic cases”.

Paget’s disease of bone have been reported as being very rare in the Chinese population (Thomas and Shepherd 1994), In 2011 an study characterized the clinical manifestations and features of 13 Chinese patients with “sporadic Paget’s disease of bone”, the finding include similar features to those reported in Western countries. The most common lesion sites were the pelvis, the femur and tibia; after those most common were the spine and skull. Most patients got elevated serum alkaline phosphatase level. Treatment with bisphosphonates was effective and within the group characterized they found the first reported SQSTM1 genetic mutation that contributes to the pathogenesis of PDB in Chinese patients (Gu et al. 2012)

1.2.6.1. Family studies

A variable percentage of patients (5-40%) have a positive family history of PDB with an autosomal dominant transmission pattern and incomplete penetrance (Haslam et al. 1998; Morales-Piga et al. 1995; Singer and Leach 2010). Although multiple families have been reported with PDB, the average number of affected individuals in these families is three. A study performed in New Zealand (Tilyard et al. 1982), identified two additional families that seemed to segregate the disease in an autosomal dominant fashion. Each family had four affected members.

A positive family history of PDB was reported in nearly 15% of patients in two large studies. In Spain, it was reported that 40% of patients had a least one first degree relative with PDB after screening with bone scans (Morales-Piga et al. 1995). In the same study, they reported that PDB seemed to be transmitted through either parent, suggesting an autosomal dominant mode of inheritance.

Linkage studies in PDB have shown evidence of genetic heterogeneity. Chromosome 18 has been extensively studied in relation to Paget's disease based on the fact that Familial expansile osteolysis (**FEO**) was linked to that chromosome. This is a rare bone dysplasia that shares some features with PDB and is also transmitted as an autosomal dominant disorder; it is caused by constitutive activation of RANK due to an 18-bp tandem duplication in the first exon of the TNFRSF11A gene (Hughes et al. 2000). FEO exhibit several histological similarities to Paget's disease such as the existence of woven bone, large osteoclasts and the presence of microcylindrical inclusions in osteoclast nuclei (Dickson et al. 1991; Wallace et al. 1989). FEO is recognized as being distinct from PDB since it has a predominantly peripheral distribution, with an onset in the second decade. There is also a progressive osteoclastic resorption accompanied by medullar expansion which leads to severe, painful, disabling deformity and a tendency to pathologic fracture. In many cases FEO is associated with early onset deafness and loss of dentition as a result of unique middle ear and jaw abnormalities. Some families with Classical PDB were linked to chromosome 18q21 but linkage studies and mutation screening have excluded the involvement of RANK in the majority of patients with PDB (Good et al. 2001). The connection between chromosome 18, FEO and Paget's disease still has not been established (Haslam et al. 1998; Hocking et al. 2000; Nance et al. 2000). On the other hand FEO has certainly be linked to chromosome 18 (Hughes et al. 1994).

Another disease that shows phenotypic overlap with PDB is **Early onset familial PDB**, also inherited in an autosomal dominant trait. Although some features are shared with classic PDB, there are several distinguishing features such as the early onset and hearing impairment occurring in the late teens or early 20s. As in the case of FEO individuals with the disease experienced premature tooth loss (second and third decades of life) (Nakatsuka et al. 2003). There are some features in common with classic PDB such as axial skeletal involvement, bone

expansion and osteosclerotic lesions. Like FEO, this disease is caused by an insertion, a mutation of the TNFRSF11A (Hughes et al. 2000; Nakatsuka et al. 2003).

Expansile skeletal hyperphosphatasia (ESH) is another metabolic bone disease, inherited as a highly penetrant autosomal-dominant that is characterized by early-onset deafness, premature tooth loss and involvement of the skeleton with progressive hyperostotic expansion of the long bones affecting the fingers, distinguishing from the other disorders due to the episodic hypercalcaemia and widespread diffuse bone involvement without focal osteolytic lesions (Ralston 2008; Whyte et al. 2000). This disease is also due to a 15bp insertion mutation in the TNFRSF11A gene (Whyte et al. 2000).

Inclusion body myopathy, Paget's disease and Frontotemporal dementia (IBMPFD) are autosomal dominant disorders characterized by 3 main features: disabling muscle weakness (in 90%), osteolytic bone lesions consistent with Paget's disease (in 51%), and frontotemporal dementia (in 32%) manifested by prominent language and behaviour dysfunction with a mean age of onset at 54 years old (Kimonis and Watts 2005; Wehl et al. 2009); In IBMPFD myopathy is the most prominent feature (occurring in 90% of the cases) and death occurs typically at a mean age of 58 years due to respiratory and cardiac failure (Ralston 2008).

The last, but not less important disease is **Idiopathic hyperphosphatasia (IHH)** or juvenile PDB. This is a rare autosomal-recessive condition characterized by a generalized increase in bone turnover, skeletal deformity, bone expansion, bone pain and an increased risk of pathological fractures. The disease is caused by a deficiency of osteoprotegerin (OPG) due to the loss of function mutation in the TNFRSF11A gene and chr8q24 (Cundy et al. 2002; Whyte 2006). The most distinguishing features include early age onset (Ralston 2008; Whyte et al. 2002). Other features include expanded and bowed extremities. Alkaline phosphatase levels are elevated in parallel with other markers of bone turnover, long bones are greatly expanded with osteoporosis and coarse trabeculations and calvaria markedly thickened with islands of increased bone density; there is premature loss of teeth, dwarfism, enormously increased turnover of subperiosteal bone (Eyring and Eisenberg 1968), progressive sensorineural deafness, kyphosis and acetabular protrusion with an increase severity during adolescence. Milder presentation have been also reported, however, a genotype-phenotype

relationship have been described, where the severity of the disease is depending of the mutation causing the disorder (Cundy et al. 2002; Ralston 2008).

1.2.6.2. Genes and Loci for PDB

The classical approach for finding the particular gene responsible for any given human disease is linkage analysis. More recently genome wide association studies (GWAS), have been used to identify disease genes. Both approaches have been used in PDB.

Linkage analysis has been used to identify the genes for familial Paget's disease. Few larger kindreds have been used for linkage analysis. One study in 1977 used haplotype data obtaining a maximum LOD (logarithm of the odds ratio of a particular locus being linked or not linked to the disease locus) score of 2.44 with 11% recombination (LOD score of 3 is considered linkage), which was considered as "suggestive" (Fotino et al. 1977). A later study in New Zealand identified two additional families in which the disease seemed to segregate with HLA in an autosomal dominant fashion (each family with four affected members). The combined linkage analysis from both studies resulted in a maximum LOD score of 3.69 with 10% recombination.

In 2001 a Genome wide linkage analysis of 319 individuals, from 62 kindreds with familial PDB (predominantly British descent) identified three chromosomal regions with LOD scores above the threshold for suggestive linkage. These were on chromosomes 2q36, 5q35, and 10p13 (where 2q36 and 10p13 had a lower LOD score). These studies indicated the presence of three susceptibility loci for PDB and identified a strong candidate locus for the disease, on chromosome 5q35 (Hocking et al. 2001).

Laurin in 2001 performed genetic linkage analysis in 24 large French Canadian families (479 individuals) in which Paget's disease was segregating as an autosomal dominant trait. After excluding linkage to the PDB2 locus, they performed a genome-wide scan on the three most informative family nuclei. LOD scores greater than 1.0 were found at 7 locations. The disorder in 8 of the families showed strong linkage to chromosome 5q35-qter, and the disease locus was designated PDB3. The same characteristic haplotype was carried by all patients in the 8

families, suggesting a founder effect. The disorder in the 16 other families, with very low conditional probability of linkage to 5q35-qter, showed linkage to 5q31 (PDB4) (Laurin et al. 2001).

Subsequently, several genetic loci (PDB1-PDB7) have been reported to predispose to late-onset PDB. Seven loci have been reported and they are referring to the following chromosomes respectively: 6p21.3, 18q21.1-22, 5q35, 5q31, 2q36, 10p13, 18q23 (Daroszevska and Ralston 2005). At present PDB3 (5q35) is the only locus where a gene, sequestosome1 (*SQSTM1*), has been identified. Mutations in *SQSTM1* have been associated with both sporadic and familial PDB in different populations, Rhodes *et al* reported that about 28.8% of families with a history of PDB have a mutation in the *SQSTM1* gene, which maps to chromosome 5 (Rhodes et al. 2008), Cundy reported a 26% of patients with familial PDB in New Zealand had disease-associated mutations in the *SQSTM1* gene (Cundy et al. 2011) and Hocking reported that *SQSTM1* mutations occurred in 15% to 40% of the pagetic individuals have an affected first degree relative (Hocking et al. 2002). At least 28 gene mutations have been identified in exon 7 and exon 8 of *SQSTM1*, most of which are in the ubiquitin-associated domain.

In a search for additional loci associated with PDB Albagha *et al* (Albagha et al. 2010) performed a genome-wide association study comparing genetic variants in patients with PDB (without *SQSTM1* mutations) to healthy individuals. In this work three different genomic regions at chromosome 1p13, 10p13 and 18q21 have been associated with PDB. In each of these regions a putative predisposition gene associated with susceptibility to PDB was proposed as shown in table 1.3.

Table 1.3: Candidates genes proposed by Albagha *et al* as predisposition genes associated with susceptibility to PDB.

Candidate genes	Chr	...encodes	Related Diseases
<i>CSF1</i>	1p13	M-CSF	Osteopetrosis.
<i>TNFRF11A</i>	18q21	RANK	Familial expansile osteolysis, Early onset PDB, ESH.
<i>OPTN</i>	10p13	Optineurin	Glaucoma
<i>PML</i>	15q24	PML	Promyelocytic leukemia
<i>RIN3</i>	14q32	Ras and Rab interactor	Cardiac tamponade, PDB
<i>NUP205</i>	7q33	Nucleoporin 205 KDa	Influenza, PDB
<i>TM7SF4</i>	8q22.3	DC-STAMP	PDB

In a subsequent study three new loci and their association with PDB were identified (Albagha et al. 2011).

Genetic studies in patients with apparent sporadic Paget disease are difficult to interpret and it is possible misclassification. Lucas *et al* (Lucas et al. 2005) found that most *SQSTM1* mutations are carried on one of two common haplotypes in patients classified as having sporadic PDB suggesting that these patients are probably misclassified. Common haplotypes suggest that these “sporadic” patients have had a family history that was unreported and were misclassified. As clinical screening of other family members is not always possible, these individuals may have a hereditary PDB.

1.2.6.3. Pro/Cons of linkage and GWAs in the identification of genetics variants

After almost 2 decades of relatively unsuccessful attempts to identify genes for complex genetic diseases by means of linkage mapping, the application of genome-wide association studies (GWAs) over the past 5 years has identified a wealth of novel disease associations. Where GWAs investigate common diseases using case-control or cohort studies rather than extended or nuclear families for rarer diseases. Microarray technologies enable fast and accurate genotyping of millions of single nucleotide polymorphisms (SNPs) in a short time. In contrast, genome-wide family-based linkage studies had much lower resolution, with standard panels including only hundreds of microsatellite markers. The success of the GWAs has been overwhelming; but unexpectedly, many of the identified associations do not map to genes but to genes deserts, and the biology underlying these discoveries is rarely immediately apparent; that just show the importance and value of functional studies following the detection of association in GWAs (Ziegler and Sun 2012).

Specifically as mention on an study in 2015, previous genome-wide association studies identified a locus on chromosome 14q32 tagged by rs104986 which was significantly associated with susceptibility to PDB in several European populations. Fine-mapping of the candidate locus confirmed that the association was confined to *RIN3* gene, some variants have been also described which are over represented in case compared with controls, the biology underlying these discoveries is rarely immediately apparent as we said before, the link to the disease translate so far as a susceptibility to PDB mediated by a combination of common and rare coding variants in *RIN3* (Vallet *et al.* 2015).

The success of GWAs using a large number of unrelated individuals has only made possible by the advancements in microarray technology. However, it also required new developments in statistical methodology, novel study designs and new methods for analysis to complement the established study designs and methods because of the complexity in genetics.

It is clear that the future success of genetic studies will require well-defined, interpretable, and commonly accepted phenotype definitions because the complete catalog of the human genome

sequence does not tell us what that sequence does. Finding genomic variants that differ between those who have and those who do not have disease will not automatically help us to understand and treat the many diseases to which humans are prone; neither will do with Paget's disease of bone. We should clarify that the common disease-common variant GWAs approach fails to identify rare variants, although we know that "multiple rare variants in disease gene can play an important role in disease susceptibility" (Ziegler and Sun 2012).

Still family information is relevant to refine the genetic model and for estimation of disease risk. The large pedigree design is practical, efficient, and well suited for investigating rare variations. Also unless the sample sizes are very large, rare functionally relevant variants with large effect size will be difficult to detect in population-based studies (Wilson and Ziegler 2011).

1.3. *SQSTM1* and PDB

There have been many advances in understanding the role of genetic factors in the pathogenesis of PDB over recent years and several genetic variants have been identified that predispose to the disease (Ralston et al. 2008). The most important of these is *SQSTM1* which was initially identified in patients with familial PDB by positional cloning (Laurin et al. 2002). Subsequent studies showed that *SQSTM1* mutations occur in up to 40% of patients with familial PDB and up to 10% of patients with “sporadic” PDB (Hocking et al. 2002).

Mutations of *SQSTM1* are thought to play a causal role in the pathogenesis of PDB (Ralston et al. 2008). They have been found to segregate with the disease in families and have an overall frequency of between 5-10% in PDB patients as compared with less than 0.07% in unaffected controls (Eekhoff et al. 2004a; Hocking et al. 2004; Laurin et al. 2002). It should be noted however, that at least one subject has been described who had not developed PDB by the seventh decade despite being a carrier of the P392L mutation of *SQSTM1* indicating that penetrance is incomplete (Morissette et al. 2006). Previous studies have shown that patients with mutations of *SQSTM1* tend to have an earlier age of onset than those without mutations (Eekhoff et al. 2004a; Hocking et al. 2004; Laurin et al. 2002); but the relationship between *SQSTM1* mutations and clinical outcome of PDB is still unclear.

As the disease is focal, rather than systemic, additional local factors may in some cases act as disease triggers, although the nature of these is unclear. Adding the occurrence of somatic mutations, mutations that are present in bone but not in circulating blood. Somatic mutations are often found in the pagetic bones of patients with sporadic PDB and pagetic osteosarcoma (Merchant et al. 2009).

Mutations in the Sequestosome 1 gene (*SQSTM1*) are an important cause of PDB. Most of the mutations identified until now, affect the ubiquitin-associated (UBA) domain of *SQSTM1*, a region of the protein that binds non covalently to ubiquitin (Layfield and Hocking 2004). Most of the mutations described until now are resulting in either loss of function, truncation

or deletion of the ubiquitin binding-associated (UBA) domain (Fig.1.10 and Fig.1.11). At present, around 30 different mutations have been identified in the gene encoding sequestosome 1 (Table 1.4). The abnormalities on UBA domain may be responsible for the elevated osteoclastic formation and bone resorption associated with PDB (Yip et al. 2006).

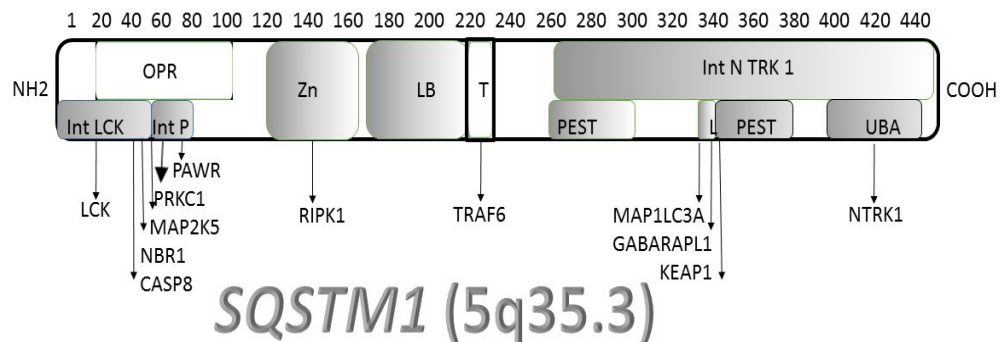


Figure.1.10: Sequestosome 1 Gene structure; *SQSTM1* (*SQSTM1*)/p62 (440 amino acids). (5q35.3). Interaction with LCK (aa 1-50). Int.: Interaction with PAWR (aa 50-80). Zinc finger (ZZ-type) (aa 122-167). LB; LIM-binding (aa 228-233). PEST (aa 266-294). Interaction with NTRK1 (aa 269-440). L: LIR motif (SGGDDDWTHLSS) (aa 332-343). PEST (aa 345-377). UBA (aa 389-434).

The protein encoded by the human *SQSTM1* gene, is a cytoplasmic scaffolding/adaptor protein and has been implicated in diverse cellular functions (Geetha and Wooten 2002) as the modulation of potassium channel function, control of transcriptional activation (Ciani et al. 2003), protein recruitment to endosomes and in close relation with PDB, control of NFκB signalling and autophagy (Layfield and Hocking 2004).

Some of the mutations, denoted A390X, K378X, E396X, Q400X, E394X, Y383X, D423X, and D408X, disrupt translation of the p62 UBA domain via introduction of stop codons, resulting in premature termination of the protein (Fig. 1.11). Recent evidence indicates that patients harbouring truncating mutations display more extensive disease compared to those

with point mutations (Hocking et al. 2004; Rea et al. 2006). The most common mutation is P392L, substitution of proline by leucine on position 392, modifies the structure of the UBA domain by extending the N terminus of helix 1, do not affect multi-ubiquitin chain binding by p62 (Ciani et al. 2003).

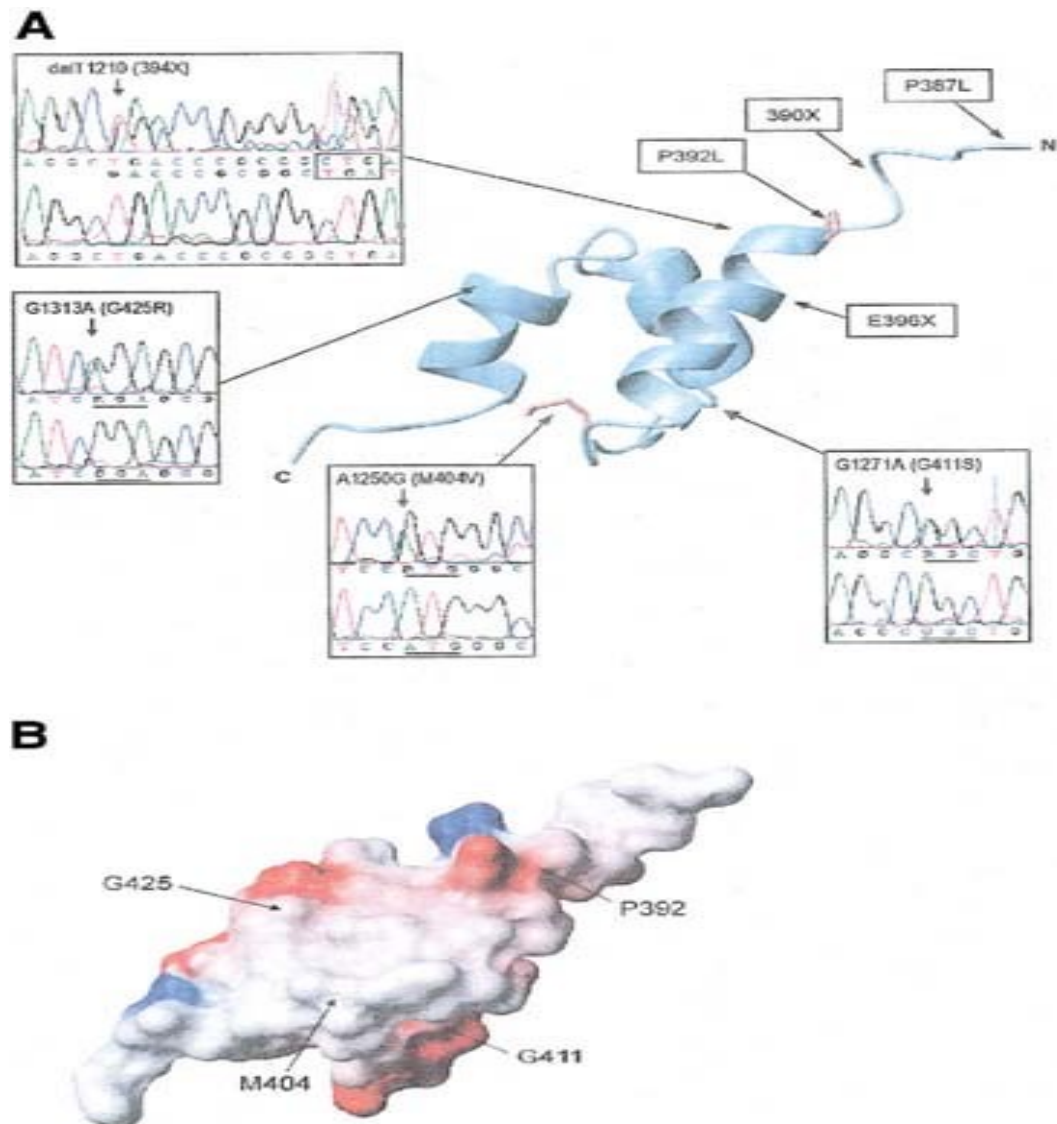


Figure.1.11: PDB causing mutations of *SQSTM1* in relation to UBA domain structure. (A) Sequencing traces for novel PDB causing mutations in relation to the ribbon structure of the UBA domain. (B) Surface representation of the *SQSTM1* UBA domain showing the hydrophobic patch (white) and some mutations (Hocking et al. 2004).

Table 1.4: Mutations described on exon 7 and 8 of the UBA domain. Other missense mutations are label as other m.

Nucleotide	Protein	Mutation type	Author/Notes
T1085A	S349T	missense	(Michou et al. 2011)
C1090T	P364S	missense	(Rea et al. 2009)
C1205C	A390X	truncating	(Beyens et al. 2006)
IVS7+1G>A		truncating	(Hocking et al. 2002)
A1132T	K378X	truncating	(Rea et al. 2006)
C1215T	P392L	missense	(Laurin et al. 2002)
1225insT	E396X	truncating	(Hocking et al. 2002)
delT1210	L394X	truncating	(Johnson-Pais et al. 2003)
C1238T	Q400X	truncating	(Visconti et al. 2010)
A1250G	M404V	other m.	(Hocking et al. 2004)
T1251C	M404T	other m.	(Eekhoff et al. 2004a)
G1271A	G411S	other m.	(Hocking et al. 2004)
T1290A	L417Q	Other m.	(Michou et al. 2011)
T1311G	I424S	other m.	(Visconti et al. 2010)
G1313A	G425R	other m.	(Hocking et al. 2004)
1348insC		extra 48 aa	(Visconti et al. 2010)
C1200T	P387L	other m.	(Johnson-Pais et al. 2003)
1215delC	L394X	truncating	
C1277T	L413F	other m.	(Collet et al. 2007)
C1182T	A381V	other m.	
T1235C	S399P	other m.	(Eekhoff et al. 2004a)
A1241G	M401V	other m.	(Gennari <i>et al.</i> 2010)
C1320A	A427D	other m.	
T1085A	S349T	other m.	(Michou <i>et al.</i> 2010)
C1209T	A390V	other m.	

T1290A	L417Q	other m.
T1046A	D335E	other m.
C1190A	Y383X	Truncating
T1229G	S397A	other m.
1307insT	D423X	Truncating
G1312A	G425E	other m.

(Falchetti *et al.* 2009)

SQSTM1/p62 is an adapter protein which binds ubiquitin and regulates signalling cascades through ubiquitination. Ubiquitin is best known for its function in targeting proteins for degradation by the proteasome (Sun and Chen 2004). This multifunctional protein regulates the activation of nuclear factor kappa-B (NFkB) by TNF-alpha, nerve growth factor (NGF) and interleukin-1. More recently, a role for *SQSTM1/p62* in macroautophagic removal of intracellular protein aggregates has also been proposed. Studies involving the cellular depletion of *SQSTM1/p62* have indicated a need for its association with LC3 and the aggregate proteins in order to facilitate correct formation of the autophagosome. The protein *SQSTM1/p62* was initially identified as a phosphotyrosine-independent ligand of the Src homology 2 (SH2) domain of p56^{lck}, *SQSTM1/p62* functions to link signalling molecules such as RIP, TrkA, and TRAF6 to atypical protein kinase C (aPKCs) in response to various stimuli, including tumour necrosis factor- α , interleukin-1, NGF, and RANKL. Earlier studies have shown that *SQSTM1/p62* non-covalently binds ubiquitin at its C-terminus, suggestive of functioning as a storage compartment for ubiquitinated proteins. In addition, *SQSTM1/p62* binds specifically to lysine-63 (K63)-polyubiquitinated substrates, acting as a putative ubiquitin chain-targeting factor that shuttles these substrates for proteasome degradation via its UBA domain.

SQSTM1/p62 has been implicated in the regulation of osteoclast formation; deletion of the UBA domain of *SQSTM1/p62* potentiates RANKL-Mediated Osteoclast formation and multinucleation, it also disrupts *SQSTM1/p62* co-localization with TRAF6 (Yip et al. 2006). Additionally *SQSTM1/p62* appears to function as an adaptor recruiting CYLD to TRAF6 and CYLD negatively regulated RANK signalling by inhibiting TRAF6 ubiquitination, meaning

that CYLD mediate the negative signalling function of SQSTM1/p62 by deubiquitinating TRAF6 (Jin et al. 2008).

Mice lacking SQSTM1/p62 exhibit modified osteoclastogenesis *in vivo* and reduced IKK activation and nucNF- κ B) translocation. The protein functions in concert with TNF receptor-associated factor 6 to mediate activation of NF- κ B in response to upstream signals. Alternatively spliced transcript variants encoding either the same or different isoforms have been identified for this gene.

In the study made by Yip, K, H, M *et al*, over expression of the SQSTM1/p62 UBA domain deletion mutant (p62 Δ UBA) significantly boosted osteoclastogenesis *in vitro* compared to controls. Over expression of p62 Δ UBA increased the formation of abnormally large multinucleated osteoclasts and resorption of bone (as in PDB) and also increased RANKL-induced activation of NF κ B, NFAT, and ERK phosphorylation. Deletion of the SQSTM1/p62 UBA domain reduced the association of SQSTM1/p62 with TRAF6 in the proteasomal compartment. These observations suggest that the UBA domain encodes essential regulatory elements required for receptor activator of NF- κ B ligand-induced osteoclast formation and bone resorption that may be directly associated with the progression of PDB (Yip et al. 2006).

1.4. Environmental Triggers for PDB

Both genetic and environmental factors are thought to be involved in the pathogenesis of PDB. The environmental factor which has been most widely studied is paramyxovirus infection (Roodman and Windle 2005) although the role of paramyxovirus infection in the pathogenesis of PDB remains controversial (Rima et al. 2002). Other potential risk factors for PDB include low calcium intake during childhood (Siris 1994), vitamin D deficiency during childhood (Barker and Gardner 1974), repetitive mechanical loading of affected bones (Solomon 1979) and environmental exposures to toxins (Lever 2002).

The changes in severity and also occurrence of the disease that have been found coupled with variable penetrance of Paget's disease within families, demonstrating that an environmental factor may be operative. The concentration of cases in Lancashire, England (Barker et al. 1980) and in Australia (Gardner et al. 1978) has been used to suggest an important environmental factor in the aetiology but could also be due to a "founder effect" with clustering of people who carry predisposing genetic variation. Additionally high prevalence areas have been seen and reported in Spain and Italy (Corral-Gudino et al. 2012; Gennari et al. 2005; Guadagnoli et al. 2008).

The focal nature of Paget's is still unexplained, which has led to the hypothesis for an infectious aetiology in Paget disease of bone, **slow virus infection** theory. Also the detection of somatic origin mutations in sporadic PDB and in cases with pagetic osteosarcoma suggests a modification of the pattern (Merchant et al. 2009), which could be explained by the viral infection. It is not possible to completely explain the etiology of PDB by SQSTM1 alone. If SQSTM1 mutations were sufficient to initiate PDB, all patients with the inherited form of the disease would develop the same form of the disease than the sporadic form of the disease. Moreover are clear differences between inherited or familial form of the disease and the sporadic form of the disease, leading to the hypothesis of other factors involved in the presentation of the disease. Perhaps the occurrence of viral infections could affect the aging bone or osteoclast precursors, or even stimulation of the immune system for long periods of

time could modify its functioning making them permissive to the clinical expression of PDB (Merchant et al. 2009).

1.4.1. Mechanical Loading

The skeletal changes in PDB disease could have unusual distribution and the reason for a variation on the sites affected is unknown. A number of cases have been reported where mechanical loading may determine the sites affected. In 1969 a case with extensive Paget's disease was described, where a leg paralysed by poliomyelitis was described as free of Paget's (Barry 1969). Also in 1979 a short report described a case of a champion billiards player who developed Paget's disease in his hands with a pattern that clearly corresponded to lines of pressure created by aligning his cue (Solomon 1979). Also in 1979, Gasper described in a letter a case of a 79 years old lady with PDB in her right leg associated with her occupation as a maker of wrist bands on a machine that she used to operate pushing with her right foot a foot operated lever; in this particular case the absence of PDB in her left leg, suggested that the repeated physical trauma triggered the onset of Paget's disease in the affected site (Gasper 1979).

On a recent review of the literature, not new information have been added to the influence of mechanical loading, recently a study tried to characterize the alterations to the structure and composition as well as the mechanical properties of bone from healthy patients and those with PDB. The study indirectly confirm that mechanical loading may not be a relevant factor on the pathology of the disease. The alterations to the structure in PDB produce bowing/deformities, namely from the low mineral content, but may also improve the mechanical integrity of the tissue by promoting plastic deformation to stop the growth of cracks, leading to the presence of stable fissure fractures characteristic of the disease (Zimmermann et al. 2015).

1.4.2. Nutrition

On 1994, Siris carried out an interesting epidemiological study based on questionnaires completed by 864 pagetic patients and 500 controls; where one of the findings was that the dietary **calcium intake** in cases was much lower than in controls. The calcium intake in this particular study was assessed by milk intake during childhood (Siris 1994). Also linked with the diet, the broad geographical association between the current distribution of PDB and that of Rickets at the beginning of the century leads to the hypothesis that another trigger for PDB is **vitamin D deficiency** in childhood. The secular trends for a decreased incidence of PDB appears to follow the secular trends of rickets disease, but the evidence for an association between PDB and vitamin D intake during childhood has not yet been confirmed (Barker and Gardner 1974)

1.4.3. Environmental Toxins

Another possible trigger of the disease that has been suggested is **exposure to environmental toxins**. In 1974 a survey in PDB showed a cluster of towns in Lancashire with the highest prevalence of PDB. They were former cotton mill towns and a link was reported between the use of calcium arsenate pesticide and the high-prevalence findings. The suggestion in that study was that geochemical arsenate was widespread in the environment and may account for geographic variations in PDB prevalence (Lever 2002). As today, it is not recent evidence of the environmental toxins been a trigger for PDB, not further studies had reported cases or further studies on the subject.

1.4.4. Other Risk factors

Many environmental factors have been suggested, including rural life style (Merlotti et al. 2005). In Italy they suggested a link between PDB and contact with animals in rural districts. The study of Merlotti *et al.* showed a correlation between having pets, living in rural areas and being in contact with pigs with the prevalence of PDB; also a correlation was shown concerning contact with cattle. In addition, the contacts with bovine cattle, the consumption

of animal viscera has been linked with PDB aetiology (Lopez-Abente et al. 1997; Merlotti et al. 2005). More recently Gennari *et al* (Gennari et al. 2010) reported on a study with patients from Campania-Italy that PDB patients exposed to animal contacts showed an increased number of affected sites and a higher prevalence of familial disease than patients without animal contact.

1.4.5. Infections-Viral Aetiology

As mentioned above, epidemiological studies indicate that environmental factors may also play a role in regulating susceptibility of the disease or perhaps even a link with the severity or sites affected. A viral cause for PDB was first proposed in the 1970's when electron microscope studies pointed to the occurrence of inclusions in the osteoclasts of patients with Paget's disease which were thought to resemble viruses (Rebel et al. 1974).

After that other studies confirmed the occurrence of nuclear inclusions, consisting essentially of striated filaments around 110 to 150 Å in diameter, often organized in bundles and sometimes in paracrystalline arrays (Harvey et al. 1982; Howatson and Fornasier 1982; Mills et al. 1980; Mills and Singer 1976; Rebel et al. 1974); Viral budding-like structures containing these inclusions were found at the peripheral cytoplasm or cell processes in the ruffled border of some pagetic osteoclast of some fresh tissues from patients with Paget's disease (Abe et al. 1995). While inclusions are typical of Paget's disease, they are not specific and similar structures have been found in the osteoclasts of patients with osteopetrosis (Mills et al. 1988) and pycnodysostosis (Beneton et al. 1987) and also in macrophages from patients with primary oxalosis (Bianco et al. 1992) and also in osteoblasts from patients with otosclerosis (McKenna et al. 1986; McKenna and Mills 1990).

Previous studies have reported the presence of measles RNA by *in situ* hybridization and also the presence of measles antigens by immunostaining in Pagetic tissue (Basle et al. 1986; Rebel et al. 1980a). **Respiratory syncytial virus** antigens have been identified by immune staining in bone sections and cultures from PDB patients (Mills et al. 1981; Mills et al. 1984;

Pringle et al. 1985). Simian virus 5 (Basle et al. 1985) and parainfluenza virus type 3 antigens have been also detected by fluorescent antibody technique.

In 1985 an apparent relationship between PDB and dog ownership (O'Driscoll et al. 1990; O'Driscoll and Anderson 1985) was reported. Keeping in mind that **canine distemper virus** (CDV) is also a paramyxovirus and it is closely related to measles, this led to the hypothesis that CDV may be a trigger for the disease. In 1991 CDV RNA was reported to be detected in osteoclasts, osteoblasts and osteocytes in PDB cases, but not in controls, by *in situ* hybridisation and by reverse transcription PCR (Gordon et al. 1991; Gordon et al. 1992). Further studies confirmed these findings (Gordon et al. 1993).

Evidence for **measles** virus antigens in the osteoclast of pagetic patients was found using antimeasles antisera and sera from patients with subacute sclerosing panencephalitis (Basle et al. 1979; Basle et al. 1981; Rebel et al. 1980a; Rebel et al. 1980b). Also Barbara Mills reported in 1984 that antigens of two viruses, measles and respiratory syncytial virus were found in the same osteoclast (Mills et al. 1984). In 1985 Pringle *et al* had surveyed the presence of neutralising and immunoprecipitating antibodies to respiratory syncytial virus and parainfluenza virus type 3 in 177 patients attending a bone disease clinic. Thirty-six of the patients had confirmed PDB. All the samples tested possessed neutralising activity for both viruses but not significant differences were reported in this study between Paget and non-Paget patients. In summary the serological results in this study highlight that persistent infection with respiratory syncytial virus can occur (Pringle et al. 1985).

In 1996 Reddy *et al* detected measles virus nucleocapsid transcripts in circulating blood cells from patients with Paget's disease by RT-PCR (Reddy et al. 1996). It was later reported that measles virus nucleocapsid protein gene transduction to normal human osteoclast precursors results in formation of osteoclast that were reported to have pagetic phenotype (Kurihara et al. 2000). Also, measles virus infection of osteoclast precursors from CD46 transgenic mice formed osteoclast with a pagetic phenotype *in vitro* (Reddy et al. 2001). Most recently, a team of researchers lead by the University of Pittsburgh School of Medicine, published results based on the bone marrows of 12 pagetic patients and 8 controls, finding viral proteins (MVNP) in at least 8/12 of the pagetic samples (Kurihara et al. 2011).

All previous positive reports, suggested that there may be an association between Paget's disease and persistent viral infection following an initial focal infection 20 to 30 years previously. For the virus to remain present after this length of time following the original infection any number of mechanisms may be involved to enable the virus to persist and escape detection by the immune system. We can mention as an example, the chronic infection of the brain caused by measles **subacute sclerosis panencephalitis (SSPE)**, where the virus persist by producing either incomplete or mutated viral proteins (Cattaneo et al. 1988). Another example is the chronic necrotising mumps encephalitis where it is the host failure to produce an adequate immune response which explains a low grade infection in the brain.

On the other hand, osteoclasts are part of the monocyte/macrophage system and share many characteristics and functions. As the mononuclear phagocytic system is one of the primary defences against infection, elimination of viruses can also play a role on the persistence or latency of the virus and monocytes and macrophages can harbour virus, then osteoclast might possibly act as reservoirs, permitting paramyxovirus replication, latency and persistence (Gordon et al. 1991; Ohmann and Babiuk 1986).

Immunosuppressive measles virus V protein is able to suppress NF- κ B activity by preventing nuclear translocation of p65 (Schuhmann et al. 2011). Measles virus P protein is able to suppress NF- κ B by up-regulation of ubiquitin-modifying enzyme A20 (Yokota et al. 2008); also canine distemper virus induces human osteoclastogenesis through NF- κ B and SQSTM1/P62 activation (Selby et al. 2006). All the reported interactions open the possibility of regulation of bone remodelling via viral infection in different pathways.

In contrast, there have been several studies where the results do not support the hypothesis that measles or other paramyxoviruses play a role in the pathogenesis of Paget's disease (Ralston et al. 1997; Ralston and Helfrich 1999). Birch also failed to find paramyxovirus RNA in cultures of pagetic bone-cells and in pagetic bone (Birch et al. 1994). Absence of measles virus and canine distemper virus transcripts in long-term bone marrow cultures from patients with PDB was reported by Ooi (Ooi et al. 2000). Again in 2007, Mathews *et al* failed

to detect measles virus RNA, by RT-PCR in bone cells cultures from patients with PDB (Matthews et al. 2007; Matthews et al. 2008).

Many of the reported studies have been done in cultured cells. However Helfrich in 2000 failed to find evidence of measles or canine distemper viral sequences, using RT-PCR, *in situ* hybridization and immunocytochemistry in a wider selection of samples including bone biopsy specimens, bone marrow and peripheral blood mononuclear cells. Also they reported that the inclusion bodies, as described previously in Paget's disease, differed in appearance from those in Subacute Sclerosing Panencephalitis (SSPE) (Helfrich et al. 2000).

The conflicting results may be due to the differences in samples, techniques, sensitivity and specificity. In 2007 a multicentre blinded analysis by RT-PCR was applied to the detection of paramyxoviruses in PDB samples and control material. This study showed no evidence of viral mRNA in PDB samples, it also showed that one laboratory that previously had detected CDV mRNA in PDB samples, also detected CDV RNA in control (negative) samples. The results of this study suggested that the technique used was not the best technique for detection due to the high-sensitivity which varied from lab to lab; also there is a risk of contamination which may have explain why positive results occurred in some studies (Ralston et al. 2007).

1.4.5.1. *Paramyxovirus*

The *Paramyxoviridae*, have been the most widely studied family of viruses related to PDB. They are pleomorphic, enveloped negative strand RNA viruses which share a high degree of genetic and structural homology in the order *Mononegavirales*. The family is divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*, according to several features such as morphology, genomic organization, role of the encoded viral proteins and the sequence relationship between these proteins (Loney et al. 2009).

In this thesis we are going to focus on the subfamily *Paramyxovirinae* which consist of five genera: *Respirovirus*, *Morbillivirus*, *Rubulavirus*, *Avulavirus* and *Henipavirus*. Some of the well-studied viruses belonging to this subfamily include human parainfluenza virus types 1 and 3 (*Respirovirus*), parainfluenza virus 5 and **mumps virus** (*Rubulavirus*), **measles virus**

and **distemper virus** (*Morbillivirus*), and **human respiratory syncytial virus** (RSV) a member of the subfamily *Pneumovirinae*.

Paramyxoviruses contain no segmented negative-strand RNA genomes containing 6-10 tandem linked genes. Viral mRNAs are transcribed monocistronically, meaning one protein being expressed from a single mRNA, with the exception of the P gene.

Viral RNA is encapsulated with nucleocapsid (N) protein which is associated with a polymerase complex composed of phosphoprotein (P) and large (L) protein. The virion contains a lipid bilayer envelope that is derived from the plasma membrane of the host cell. The envelope is studded with glycoproteins necessary for virus egress, attachment, and entry and is lined internally by the matrix protein (M).

Structural investigation of paramyxovirus biology has proven difficult, due to disordered regions in some of their proteins, conformational flexibility in the nucleocapsid, and the pleomorphic nature of the paramyxovirus virion. An example is found in measles virus where the nucleocapsid protein has been found to bind several viral and host proteins (Loney et al. 2009). This is linked to the C-terminal domain of the measles virus nucleoprotein which is intrinsically disordered and folds upon binding to the C-terminal moiety of the phosphoprotein (Longhi et al. 2003). Paramyxovirus virions are highly pleomorphic and are shown to range in diameter from approximately 120 to 450nm in diameter (Terrier et al. 2009).

In 1994, a new member of the family *Paramyxoviridae* isolated from fatal cases of respiratory disease in horses and humans was shown to be distantly related to morbilliviruses and provisionally called equine morbillivirus. To facilitate characterization and classification, the virus was purified, viral proteins were identified, and the P/V/C gene was cloned and sequenced (Wang et al. 1998). The accessory proteins expressed from the P/V/C gene are major factors in the pathogenesis of the viruses, because of their ability to disrupt various facets of type I interferon (IFN) induction and signalling. Most of the paramyxoviruses share the ability to antagonize innate immunity by blocking IFN induction and the Jak/STAT pathway. However, the manner in which the accessory proteins work differs among viruses.

1.4.1.2 Measles

Amongst the paramyxoviruses the most studied in association with PDB had been measles virus. Ultra structural and immunological studies suggested measles or measles-related virus as the main agent involved in the pathogenesis of PDB. It has been reported that the expression of measles virus nucleocapsid gene in mice, with or without a mutation on the SQSTM1/p62 gene (P392L), develops pagetic like osteoclasts and increased Interleukin 6 levels in bone marrow dependent on p38MAPK activation. The same group reported that mice co-expressing measles virus nucleocapsid gene and P392L mutation on SQSTM1/p62 develop dramatic Paget's-like bone lesions (Kurihara et al. 2011) suggesting that measles virus plays a key role in PDB.

Interestingly, the potential of measles virus to interfere with some pathways in close relationship with bone formation, bone cells and bone remodelling has been reported. The key players of the measles virus-mediated immune evasion, are three phosphoprotein gene products P, V and C. By a process called RNA editing, additional G is inserted into the mRNA of the phosphoprotein gene transcript producing the V protein (Cattaneo et al. 1988). Measles virus essential P protein and the accessory proteins V and C appear to prevent NF- κ B – dependent gene expression by retaining p65 in the cytoplasm (Schuhmann et al. 2011). It has also been reported that measles virus P protein suppresses Toll-like receptor signal through up-regulation of ubiquitin-modifying enzyme A20 (Yokota et al. 2008).

The annual incidence of measles has been reported as around 100 cases from 1998 to 1999 in the USA, but now is greatly reduced by the MMR vaccination program; this is an incidence rate of approximately 1 in 2,720,000; by extrapolation 96 per year, 8 per month, and 2 per week. Corresponding figures in Canada were 6.5 cases per 1,000,000 people for 2000, and two/million in Australia for 2002. After the introduction of the vaccine in Spain, in 1978, the incidence decreased from 4290 cases/million in 1977 to 1.8 cases/million in 2002 (Mosquera et al. 2005). The World Health Organization reported that the global figures for 2009 were 222,408 reported cases and 164,000 estimated deaths in 2008. Measles vaccine was introduced in the United Kingdom in 1968 for the vaccination of children between 1 and 2 years of age.

Initially the coverage was poor and during the 1970s, fewer than 60% of children were vaccinated by the age of 2. In recent times vaccination has risen to almost 80% coverage but measles still continues to circulate and cause substantial morbidity and mortality (Miller 1989).

Indeed measles has recently reappeared in the United Kingdom, with 449 confirmed cases to the end of May 2006 compared with 77 in 2005, and the first death since 1992 (Perviz and Eithne 2006). In 2008, measles was declared endemic in the UK, meaning that the disease was sustained within the population. This was the result of a decade's low MMR vaccination rates, which created a population of susceptible children who could spread the disease. In May 2008. A British 17 year old with an underlying immunodeficiency dies of measles (as reported by Smith Rebecca, Medical Editor at the Telegraph. 20th June 2008. Retrieved 21st April 2013). An outbreak centered on the Swansea area of Wales started in November 2012; as of 22nd April there have been 886 cases (NHS WALES. Public health Wales Outbreak data November-July 2013).

Vaccination may eventually eradicate measles. However, controversy regarding the combined vaccine measles, mumps, rubella (MMR) vaccine, has led to reduced vaccination coverage in some countries during the last decade. In 2010, around 30,367 cases were reported from across the 32 European countries reporting to euvac.net (72% from Bulgaria, and 17% from France), and 21 measles related deaths (EUVAC annual measles report 2010. http://www.euvac.net/graphics/euvac/pdf/annual_2010.pdf . After the MMR vaccine negative publicity, associating the vaccine with autism and bowel disease (Wakefield et al. 1998), compliance dropped sharply in the UK, from 92% in 1996 to 84% in 2002 (Simon 2003). Measles cases continued to be reported in 2006 at incidence rates 13 times greater than 1998 levels. In 2008, measles was declared endemic in the UK, meaning that the disease was sustained within the population and the incidence rates 24 times greater than 1998 ("Confirmed cases of measles, mumps & rubella". Health Protection Agency 2007-03-22. Retrieved 2007-09-05). All this, may be the result of a low MMR vaccination rate which created a population of susceptible children who could spread the disease; also in some years susceptible adults could be an issue.

Susceptible groups in the UK include: unvaccinated children, young adults born in the UK between 1970 and 1979 (single measles vaccine) which in a few years also could be at risk of PDB, recipients of a single dose of measles containing vaccine, children aged less than 12 months who have not yet been vaccinated and immune-compromised people in whom live vaccines are contraindicated (Perviz and Eithne 2006).

The serology of measles is quite well established and can be summarized as follows: In individuals with acute measles four-fold or greater increases in measles-reactive antibodies are detectable by CF, HAI, IFA, and EIA. IgM antibodies to measles virus, the test of choice, are often detectable with onset of the rash and typically persist for 4 weeks (Helfand et al. 1997); at least 80% will be positive for measles IgM at 6 days and 100% at 16 days after onset of symptoms (Ozanne and Dhalewyn 1992). Over 50% of patients with recent measles will become IgM-negative at 4 months with the first negative results recorded around 9 weeks (Helfand et al. 1997). Maximal titers are reached between 15 to 19 days after rash onset (Helfand et al. 1997; Oshitani et al. 1997) False-positive measles IgM may happen due to rheumatoid factor (Helfand et al. 1997)(Fig. 1.10).

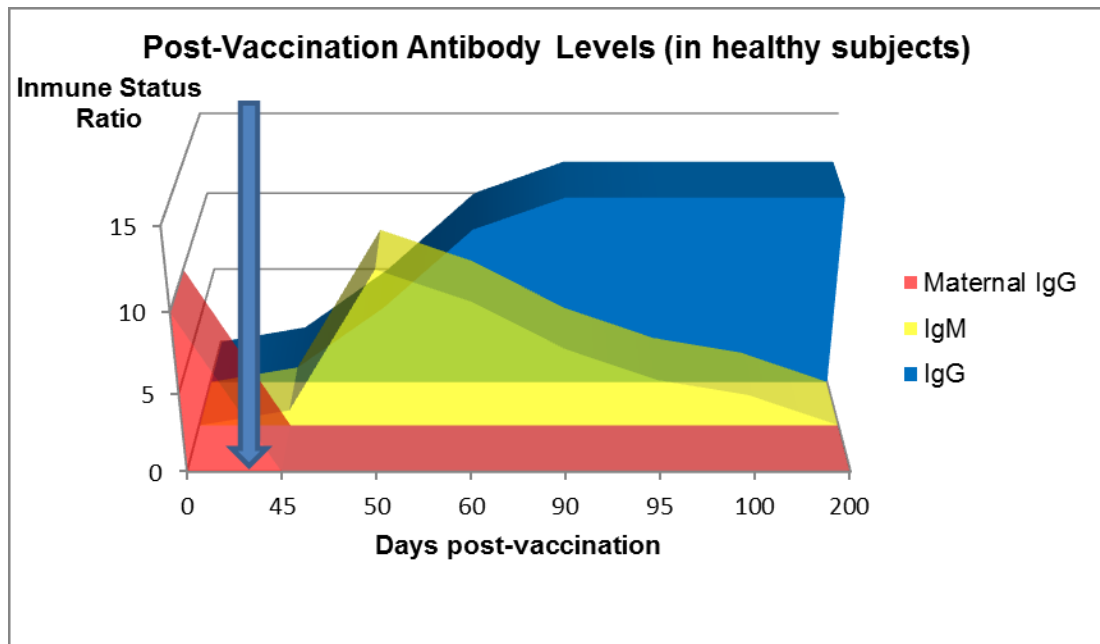


Figure.1.12. The diagram is showing a general behaviour of Immunoglobulins after vaccination, where the inside vertical line, parallel to the Y axis, is indicating the time of vaccination.

Patients with failed vaccination for measles often develop measles virus reactive IgM when infected (Ryall et al. 1996). Exposure to measles virus often leads to a secondary immune response in IgG-positive individuals but neither immune response nor prior vaccination ensures protective immunity (Ozanne and Dhalewyn 1992).

Additionally, increased intrathecal synthesis of IgG against measles virus is often seen in multiple sclerosis (Albrecht et al. 1983; Dhibjalbut et al. 1990). Also profound increases of intrathecal synthesis of measles virus-reactive IgG in sub-acute sclerosing panencephalitis (SSPE) has been reported (Patrick et al. 1990). Smaller increases are common in multiple sclerosis as part of the MRZ reaction (Felgenhauer and Reiber 1992). Increases of measles-reactive IgA and IgD in cerebro-spinal fluid (CSF) have also been reported in SSPE, as well as antibodies to mumps virus (Patrick et al. 1990; Persson et al. 1989).

The introduction of vaccination programs for measles in 1968 (Miller 1989) has been suggested as a possible reason for the reduction in incidence of PDB, but it has been recently

highlighted that this occurred too recently to account for reduction in prevalence and severity of PDB patients born in the 1930s and 1940s (Doyle et al. 2002; Poor et al. 2006a). If the theory is correct the reduction in the incidence of PDB will be seen in the years to come and onwards.

Another virus that belongs to the family paramyxovirus, and has been mentioned as a possible candidate for viral trigger on PDB is **Canine distemper**. Gordon reported canine distemper virus as the predominant paramyxovirus associated with PDB in the North West of Britain (Gordon et al. 1991; Gordon et al. 1992; Gordon et al. 1993); Distemper is most commonly associated with domestic animals such as dogs and ferrets. It is a single-stranded RNA virus (McCarthy et al. 2007) very similar to the measles virus. Canine distemper virus (CDV) seems to have appeared more recently with the first case described in 1905 by French veterinarian Henri Carre (Pomeroy et al. 2008). The virus can cause systemic infection in the host carnivore. Puppies from 3-6 months old are particularly susceptible. CDV spreads through aerosol droplets and through contact with infected bodily fluids including nasal and ocular secretions, faeces and urine. It can be also spread by food and water contaminated with these fluids. Canine distemper tends to orient its infection towards the lymphoid, epithelial, and nervous tissues (Fankhauser 1982).

1.4.1.3. Other Virus

In addition to the paramyxoviruses, other viruses have been studied for this thesis in association with PDB. That is the case for varicella-zoster virus and rubella virus.

Rubella has been chosen due to its similarity to paramyxoviruses. It is the only member of the genus Rubivirus and belongs to the family Togoviridae. It is similar to the paramyxovirus because it is a single stranded RNA of positive polarity enclosed by an icosahedral capsid. Additionally Rubella is part of the vaccine Measles, Mumps, and Rubella (MMR). Supporting our decision to include Rubella in the study is a report of bone changes after double vaccination with rubella and measles vaccine (Peters and Horowitz 1984). This article described a drastic case where a child after vaccination developed chronic arthritis and had metaphyseal changes.

Varicella-zoster virus, more commonly known as chickenpox and herpes zoster, are known clinical manifestations of infection with varicella-zoster virus. A primary infection with Varicella-Zoster Virus, usually produce the clinical syndrome known as chicken pox which is highly contagious, characterized by widely spread vesicular eruptions and fever. It most commonly affects children from 5 to 8 years of age. Herpes zoster is mainly a disease of adults, with most cases appearing in patients around fifty years of age or older. Evidence suggest that this manifestation of VZV infections results from a reactivation of virus which has remained latent in the sensory spinal ganglia after a primary infection rather than a reintroduction of the virus into the host. The capability of the virus to remain dormant in the nervous system is well known. Variola, vaccinia, varicella, and the Epstein-Barr virus have been associated with arthritis (Silverman 1976). This virus was selected as a non-related control due to his capabilities (similar to the paramyxoviruses) and the fact that belongs to a completely different family of virus, also affecting the age group we are studying.

1.6. Aims

The aim of my project is to investigate the relationship between the genotype and the phenotype of PDB patients and the interactions between genotype and environmental variables such as diet, occupation, previous musculoskeletal injury and extend of Paget's disease. The hypothesis in this case was that the different genotypes could be related with the presentation of Paget's Disease of Bone, genotypes could be associated with the severity of the disease and extend.

Additionally, another aim is to investigate the role of virus infection in PDB by serology searching for viral prints or circulating antibodies in affected patients and controls. To investigate the hypothesis where measles virus infection might act as a trigger for disease occurrence and severity in people genetically predisposed to develop PDB.

A third aim is the exploration of the possibility of mutations in other genes that have not been investigated. For the purposes of answer to the last query, I used results from the genome-wide association study and genotyped all samples available for other genes indicated in the results of the GWAS.

Chapter 2: General Materials and Methods

2.1. PRISM Patients/Samples

The study was based on participants of the Paget's disease, Randomised Trial of Intensive versus Symptomatic Management (PRISM) study (ISRCTN12989577) which was a randomised comparative trial of two treatment strategies for PDB (Langston et al. 2010).

PRISM Study population was a total of 2110 patients that were screened for possible inclusion in the study between December 2001 and June 2004 in 39 referral centres throughout the United Kingdom. In brief, the PRISM trial involved 1324 patients with PDB attending secondary care referral centres in the UK. Recruitment was based in the 39 secondary referral centres in the United Kingdom included in table 2.1. Patients were invited to take part in the study if they were known to have PDB diagnosed by standard clinical criteria (Selby et al. 2002), were considered by the attending clinician to be able to adhere to the study protocol, and have a life expectancy greater than 1 year. An overview of the trial profile is shown in figure 2. 1 and demographic characteristic of the study population are shown in Table 2.2

They were randomised to receive either "symptomatic" therapy in which treatment was administered only in patients who had bone pain or "intensive" bisphosphonate therapy in which the aim of treatment was to suppress and maintain serum alkaline phosphatase levels within the normal range by use of bisphosphonate therapy. The bisphosphonate of choice in the "intensive" group was risedronate since the trial was initiated in 2001 prior to the licensing of zoledronic acid for the treatment of PDB. The present report is based on a subgroup of 737 unrelated study participants who consented to provide a blood sample for genetic analysis.

The enrolment criteria for the PRISM study were as follow:

- ✓ PDB, diagnosed by standard clinical radiographic criteria.
- ✓ Age greater than 18 years.
- ✓ Life expectancy greater than 12 months at the time of enrolment.
- ✓ Not participating in another research project.

- ✓ Able to provide written informed consent.

In addition, non-pagetic controls were recruited. The controls were volunteers (members of staff or patients spouses) without known family history of PDB.

Table 2.1: Referral Clinical Centres

Clinical Centre	Location
Aberdeen Royal Infirmary	Aberdeen
University Hospital Aintree	Aintree
Royal National Hospital for Rheumatic Disease	Bath
Musgrave Park Hospital	Belfast
Queen Elizabeth Hospital	Birmingham
Royal Bolton Hospital	Bolton
Ninewells Hospital	Dundee
University Hospital of North Durham	Durham
Medway Maritime Hospital	Gillingham
Western Infirmary	Glasgow
Huddersfield Royal Infirmary	Huddersfield
Raigmore Hospital	Inverness
Airedale Hospital	Airedale
Leicester Royal Infirmary	Leicester
Leicester General Hospital	Leicester
Royal Liverpool University Hospital	Liverpool
Llandudno General Hospital	Llandudno
Guy's Hospital	London
King's College Hospital	London
Manchester Royal Infirmary	Manchester
The James Cook University Hospital	Middlesbrough
Freeman Hospital	Newcastle
Norfolk & Norwich University Hospital	Norwich
City Hospital	Nottingham
Robert Jones & Agnes Hunt Orthopaedic Hospital	Oswestry
Nuffield Orthopaedic Centre	Oxford
Llandough Hospital	Penarth
Derriford Hospital	Plymouth
Poole Hospital	Poole, Dorset.

Harold Wood Hospital	Romford
Oldchurch Hospital	Romford
Hope Hospital	Salford
Northern General Hospital	Sheffield
Southhampton General Hospital	Southhampton
Royal National Orthopaedic Hospital	Stanmore
The Great Western Hospital	Swindon
Queen Elizabeth II Hospital	Welwyn Garden City
Arrowe Park Hospital	Wirral
Yeovil District Hospital	Yeovil
Western General Hospital	Edinburgh

2.1.1. Additional Cohorts

On Chapter 4, for replication, we used four separate clinic-based cohorts of PDB patients. The GenePage cohort comprised 384 unrelated Italian patients with Paget's disease recruited from 13 Italian centers as previously described (Falchetti et al. 2009); the Naples/Siena cohort comprised a non-overlapping cohort of 363 unrelated Italian patients with Paget's disease recruited from northern, central, and southern Italy as described (Gennari et al. 2010); the Salamanca cohort comprised 191 unrelated patients attending a specialist clinic for Paget's disease in Salamanca, Spain (Corral-Gudino et al. 2013b), and the Western Australian cohort comprised 232 unrelated patients attending a specialist clinic in Perth (Rea et al. 2009).

2.2. Laboratory Investigations

All participants had a radionuclide bone scan prior to entering the study and the extent of skeletal involvement was assessed by counting the number of affected sites. Routine biochemistry and haematology, including measurement of total serum alkaline phosphatase (ALP) was performed at baseline and during the study according to standard techniques.

2.3. Clinical Assessments

Health-related quality of life was assessed by the SF-36 questionnaire (Langston et al. 2007a). Deformity was assessed by the attending physicians who were asked to assess whether the patient had clinical evidence of bone deformity using a three-point scale as follow: 0= no deformity; 1= mild or moderate deformity and 2= severe deformity. The presence of bone pain was recorded and physicians were asked to assess if they thought the pain was caused by PDB. Information was collected on previous fractures and whether they had occurred in affected bone; on orthopaedic surgical procedures; on the use of a hearing aid for deafness; on age at first diagnosis of PDB; and family history of PDB.

Information was recorded on whether or not the patient had previously received bisphosphonate treatment, and if so, the number of treatment courses given.

We devised a disease severity score taking several clinical features into account; giving a point for the number of bones affected on the bone scan (range 1-26); the presence of bone pain thought to be due to PDB (0= no or 1 = yes), previous fractures (0 = no or 1 = yes), previous orthopaedic surgical procedures (0= no or 1 = yes), bone deformity (0 = no deformity to 14 = severe deformity in 7 bones); and use of a hearing aid if the patient had PDB of the skull (0= no or 1 = yes). The score could theoretically range from 1 (monostotic PDB with no complications) to 43 (PDB affecting all skeletal sites with multiple complications and multiple bone deformities) (Visconti et al. 2010).

We also collected information on occupation and categorised occupations according to the UK Office for National Statistics Standard Occupation Classification 2000. Occupations were classified into three groups: those that were sedentary (for example, office workers); those that involved moderate physical activity (for example, factory workers, delivery workers, nurses and care assistants); and those that involving heavy physical activity (for example, farmers, miners and manual labourers). Milk consumption (as a surrogate for dietary calcium intake) was assessed by asking whether the patient took the equivalent of at least a glass of milk once a day; one a week or less than once a week as previously described (Visconti et al. 2010). Previous episodes of musculoskeletal injury that were serious enough for the patient to seek medical advice were recorded and patients categorised into those that had suffered such an injury (score =1) and those that had not (score =0).

2.4. DNA Extraction

DNA was extracted from patient's blood prior to the start of this project for the purpose of several studies. Extraction was carried out using the Nucleon BACC3 DNA Extraction Kit from Gen-Probe, according to the manufacturer's instructions. All samples concentrations were analysed by Pico green (using UV transparent plates and a plate reader). Subset of samples (8 out of 92) also runs on a UV spectrophotometer. The purity of the extracted DNA was determined from the OD₂₆₀/OD₂₈₀ ratio. Extracted DNA was stored in solution at -40°C. Stock DNA prepared at a concentration of 50ng/ul and working stock at a concentration of 10ng/ul, was stored at -20°C for immediate use.

2.5. Mutation Screening

For the screening of the subject for novel mutations and known *SQSTM1* mutations; Genomic DNA was extracted from peripheral blood using standard procedures, as described above. Mutation screening of *SQSTM1* was conducted on PCR-amplified fragments of DNA, on the

chromosome 5, focusing on exons 7 and 8 and the intron exon boundaries since all previously reported mutations occur in these regions (Morissette, Laurin, & Brown 2006a).

2.5.1. Primer design

Primer sequences were designed for two exons of *SQSTM1* using OLIGO 3.4 Primer Selection Software (Rychlik and Rhoads, 1989). Exon 7 is suitably short to be accommodated by one automated sequencing run so primer pairs were designed to include the entire exon 7 and the exon-intron boundaries. Exon 8 is also a suitable size to be sequenced in one run. After initial selection just a pair of primers that amplify the reading frame have been used for the continuous genotype of the exon 8. The oligonucleotides used in PCR amplification were purchased from Invitrogen and are shown in Table 2.1. These were received in a freeze-dried state, suspended in sterile water to a storage stock concentration of 100µM and diluted to a PCR working stock concentration of 10µM for PCR use and working stock concentration of 3.3µM for sequencing use.

Table 2.1: Primers used for *SQSTM1* exon 7 and 8 amplification.

PRIMER NAME	FORWARD & REVERSE SEQUENCE	Amplified Region
Exon 7	ttaaagtcacgctgggaacctgct (forward)	454 bp
	agggcaggatgctctaaaggg (reverse)	179192995
		179193448
Exon 8	tctgggcaggctcggacact (forward)	421 bp
	ccctaaatggcttcttgacccc (reverse)	179195921
		179196341

Genotyping of samples was carried out by PCR analysis of genomic DNA. All PCR reactions were performed in AB-Gene 96-well plates. The volume for each reaction was adjusted to 25ul with sterile dH₂O (10.375ul).

Table 2.2: PCR Reaction.

Volume (microliters)	Solution	Suppliers	Notes
2.5	10X Taq buffer mix	Qiagen	Catalogue#201207
2.0	dNTPs	Promega	Catalogue#U1420
5.0	Q solution	Qiagen	dATP, dCTP, dGTP, dTTP. 25µmCatalogue#201207
2.5	Primer mix	Invitrogen	1.5ul of each in a concentration of 10uM
0.125	Taq polymerase	Qiagen	Catalogue#201207
10.375	DNase/RNase free distilled water	Invitrogen	Catalogue#10977049
2.5	Genomic DNA		10ng/ul
25	Final volume for PCR reaction		

2.5.2. PCR Amplification

All the PCRs and genotyping was done by the candidate. PCR amplification was carried out in 25µl reactions (as described in table 2.2) in 96-well skirted plates (AbGene, 0.2ml per well, cat. No. AB-0600-L). 2.5µl of genomic template DNA (20ng/µl) was mixed with 10.375µl sterile distilled H₂O (GIBCO distilled water DNase/RNase free. Cat No.10977-035 500ml), 1.25µl each of forward and reverse primer (10µM), and PCR reagents from the Qiagen Taq, PCR Core Kit (2.5µl of 10x PCR Buffer containing 15mM MgCl₂; 5µl of 5x Q-Solution; 0.125µl of Taq DNA Polymerase at 5 units/µl concentration. Cat. No.201225) and 2µl dNTP Mix containing 10mM of each dNTP. PCR thermocycling was carried out in a DYAD™ DNA Engine (Peltier) and the thermocycling conditions for each primer pair are given in Table 2.

To identify any mutation on exon 7, a 454bp fragment was generated, using the primers previously described. For identification of any mutation on exon 8 a 421bp fragment was generated, using the primers as described previously.

Amplification of both fragments was performed in a MJ Research thermocycler. The thermal cycling protocol is described on Table 2.3.

Table 2.3: Thermocycling condition for PCR of exon 7 and 8. Cycle 2 is repeated for 34 cycles.

Step	Action	Temperatures	Duration
Cycle 1	Denature template	94°C	3:10min
Cycle 2 to be repeated 34 times	Denature template	94°C	0:50min
	Anneal primers	60°C	1:00min
	Extend primers	72°C	1:30min
Cycle 35	Extend primers	72°C	8:30min
	Storage	4°C	1hour

2.5.3. Agarose Gel Electrophoresis

The PCR products were visualised on a 2% agarose gel (Bioline cat# BIO41025) to confirm successful amplification of the correct product size. The gel was prepared with 1.60gr of agarose and 80ml of TBE (Tris Borate EDTA buffer) adding 8ul of SYBR safe gel stain (Invitrogen cat# S33102). As a reference for the size of the products obtained I used a low molecular weight ladder (New England Biolabs, cat# N3233S).

Tris Borate EDTA buffer was prepared from 20X TBE, which was made with 242g Tris base, 123.4g Boric acid and 14.88g Sodium EDTA (BDH) dissolved in distilled water to make a final volume of 1 litre. 5ul of PCR product was mixed with 3ul of orange G gel loading dye (0.2g orange G (Sigma) dissolved in 100ml of 30% glycerol) and later loaded into the gel wells (Fig. 2. 1).

2.5.4. DNA sequencing

The PCR products were sequenced using the same primers in both forward and reverse directions. The amplified target DNA was purified using the ExoSAP-IT enzymatic

purification system. 1µl of ExoSAP-IT was mixed with 6µl of PCR product and this mixture was incubated at 37°C for 30 minutes to accommodate enzymatic digestion and at 80°C for 15 minutes to deactivate the enzyme. This enzymatic purification allowed the removal of all molecules except target double-stranded PCR product from the working solution. This mixture was diluted 1:2 by adding 7µl of sterile H₂O, mixed thoroughly and split into two 7µl aliquots; one each for automated sequencing in the forward and reverse direction.

Amplified and labelled DNA fragments were sequenced using a MegaBACE™ 1000 DNA Sequence Analyser (Amersham). This system uses fine-bore capillaries filled with MegaBACE™ Long Read Matrix to provide a homogeneous, gelatinous medium in which molecules of different sizes can be resolved by electrophoresis. Amplified and precipitated target DNA samples were re-suspended in 8µl of Loading Solution (DYEnamic ET Dye Terminator Kit, Amersham) and vortexed. Samples were dissolved at 50°C for 2 minutes, vortexed, denatured at 95°C for one minute and placed on ice. Automated sequencing using a MegaBACE™ 1000 DNA Sequence Analyser was carried out according to the manufacturer's instructions.

2.5.5. Nucleotide Sequence Analysis

Automated sequencing data were imported into Chromas which produces a sequence chromatogram for each sample. This output was analysed manually for polymorphic variation in each exon among all members of the sample group.

The traces were analysed by Chromas-pro software and compared with the reference sequence (NC_000005.8, GI: 51511721, NCBI Entrez Gene, <http://www.ncbi.nlm.nih.gov/>).

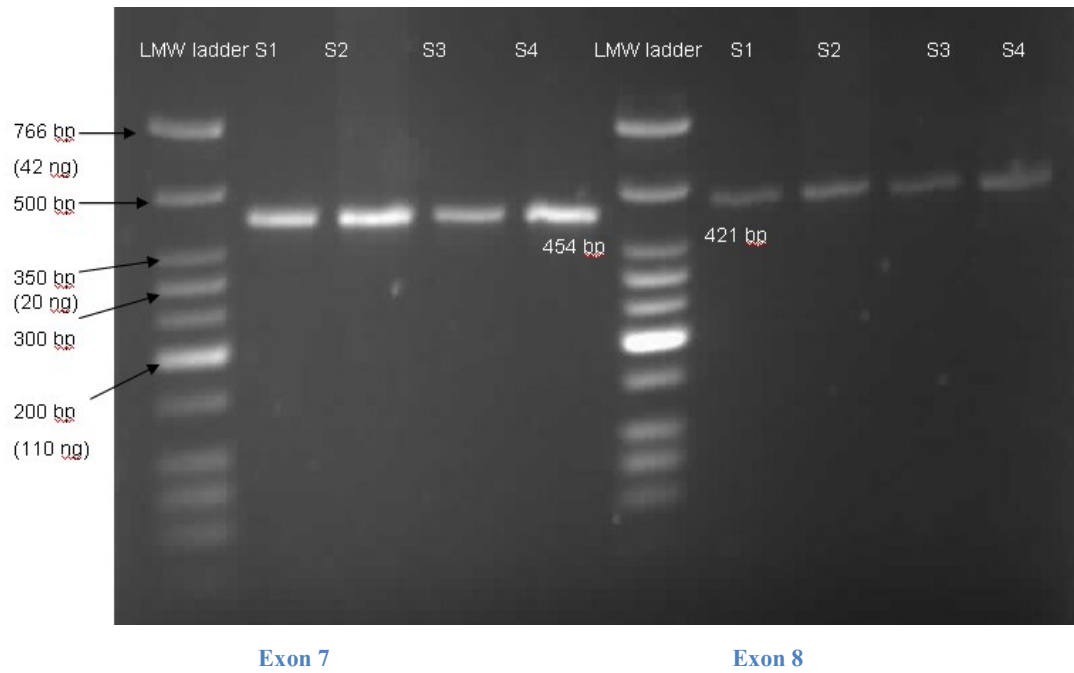


Figure 2.1: Gel electrophoresis analysis of SQSTM1 PCR. Lane 1: Low molecular weight DNA ladder; lane 2-5: Samples from patients amplified using exon 7 primers; lane 6: Low molecular weight DNA ladder ; lane 7-10: Samples from patients amplified using exon 8 primers.

2.6. Enzyme immune Linked ImmunoSorbent Assay for viral IgG (ELISA)

2.6.1. Serum for viral studies

Blood from patients was obtained on red top tubes, which contained serum clot activator. The serum was obtained by centrifugation of the whole blood at 1500g, serum removed and stored in 2x 2ml cryo-vials at -80°C. Between tests, the samples were stored at 4°C to avoid thawing and freezing.

2.6.2. ELISA

For viral testing enzyme linked immunosorbent assays (ELISA) was used for the detection and quantitative determination of IgG antibodies to Measles, Rubella, Mumps, Varicella-Zoster Virus (VZV), Respiratory syncytial virus (RSV) and Canine Distemper Virus.

For Measles virus, Mumps, Rubella and Varicella-Zoster, we used the kits mentioned in the Table 2.6.1. All these kits share the same protocol; also serum diluent buffer, Chromogen/Substrate Solution, wash buffer and stop solution are not kit lot number dependent and may be used interchangeably within the Trinity Biotech ELISA IgG assays. All the tests were performed according to manufacturer instructions.

Table 2.6.1: ELISA kits use for Measles, Mumps, Rubella and Varicella-Zoster, Respiratory syncytial virus and Canine Distemper.

Virus	Manufacturer	Catalogue number
Measles	Trinity Biotech Captia TM	2326000
Mumps	Trinity Biotech Captia TM	2325900
Rubella	Trinity Biotech Captia TM	2325300
Varicella-Zoster	Trinity Biotech Captia TM	2325600
Respiratory syncytial	MP Biomedical	071-516002
Canine Parvovirus Distemper	Biogal	11CPD200

Briefly, the samples, calibrator and controls (positive and negative) were diluted 1:21 (10ul of the sample in 200ul of diluent) in a 96 well plates specific for each ELISA. To individual wells, 100ul of the appropriate diluted calibrator, controls and patient sera was added. Each plate was incubated at room temperature (21 to 25° C) for 25min (+/- 5 min). After incubation the liquid from the wells was discarded by inverting the plate. The washing solution was added for a total of 3 washes. After the final wash, the plate was blotted on paper towelling to remove all the liquid from the wells. The conjugate was added, 100ul, and incubated for 25min at room temperature, after that another 3 washes followed. 100ul of chromogen/substrate was added and incubated at room temperature for 10-15minutes. Finally the reaction was stopped adding 100ul of stop solution (1N H₂SO₄). The plates were read on an ELISA plate reader (BIO-TEK; Synergy HT) equipped with a 450nm filter. The antibody titers were calculated for each well, by blanking all the samples, calibrators and controls; using the calibrators value to calculate the Immune Status Ratio (ISR), and for most of the virus the values are transformed into International Unit per ul (IU/ul) (Fig.2.2).



Figure 2.2: Trinity Biotech Captia Elisa.

For Respiratory syncytial virus, we use the MP Biomedical RSV IgG ELISA (catalogue number 071-516002). In this case the principle of the test was the same as the other ELISAs performed, but the protocol, was slightly different.

The assay steps were as follow: The washing buffer needed to be diluted 1 in 9 with distilled water. The samples were diluted 1 in 100 with the buffer provided (2ul in 198ul); the controls for the standard curve are ready to use and 5 standards are supplied with the kit. The samples were added to the ELISA plate (100ul) and incubated for 60 minutes. After the first incubation the plate was washed 3 times with 300ul of diluted washing buffer. The conjugate was added to each well (100ul) and incubated for 30 minutes, this was followed by 3 washes with 300ul of washing buffer. Finally 100ul of the substrate was added to each well, incubated for 20

minutes in the dark and the stop solution was added (100ul). The plate was read within 60 min. at 450nm and a reference wavelength of 620nm was used.

For Canine Distemper IgG test, ImmunoComb® Canine Parvovirus and Distemper IgG Antibody Test Kits were used. The ImmunoComb® test is a modified ELISA, which can be described as an enzyme labelled “dot assay”. In this case the assay is slightly different to the other ELISA’s performed.

The kit contains 2 main components: a comb shaped plastic card, hereafter referred to as the Comb and a multi compartment developing plate. The comb has 12 teeth, sufficient for 12 tests or samples. Each tooth contains 3 dots, one of the dots is purified parvovirus and a second dot is purified distemper virus, the upper most spot is a positive control. Purified CPV antigen is attached at the middle spot and purified CDV antigen is attached at the lowest of the 3 spots (see figure 2.3).

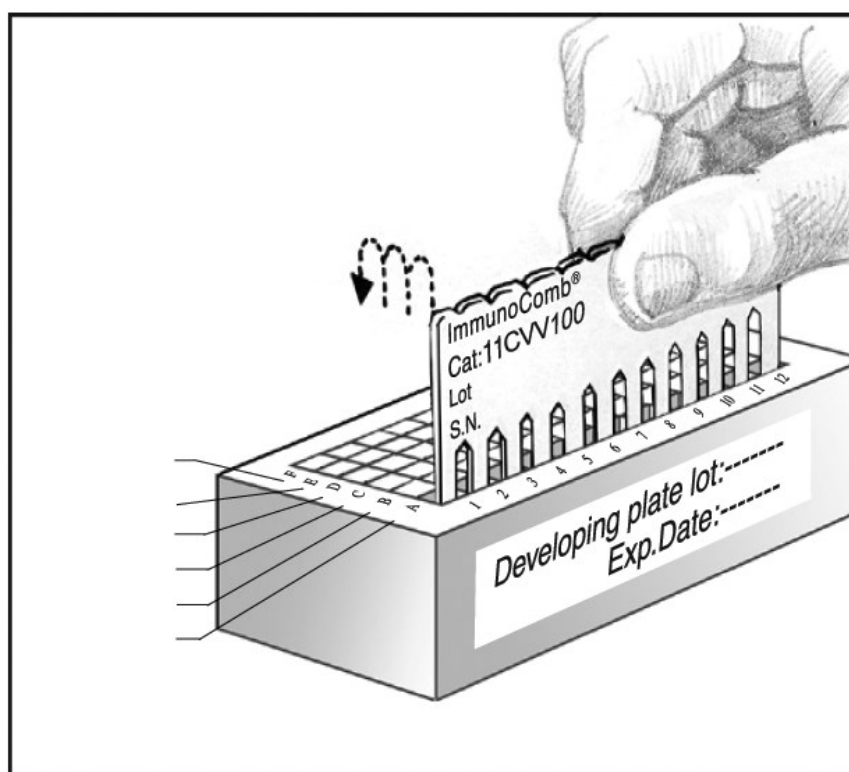
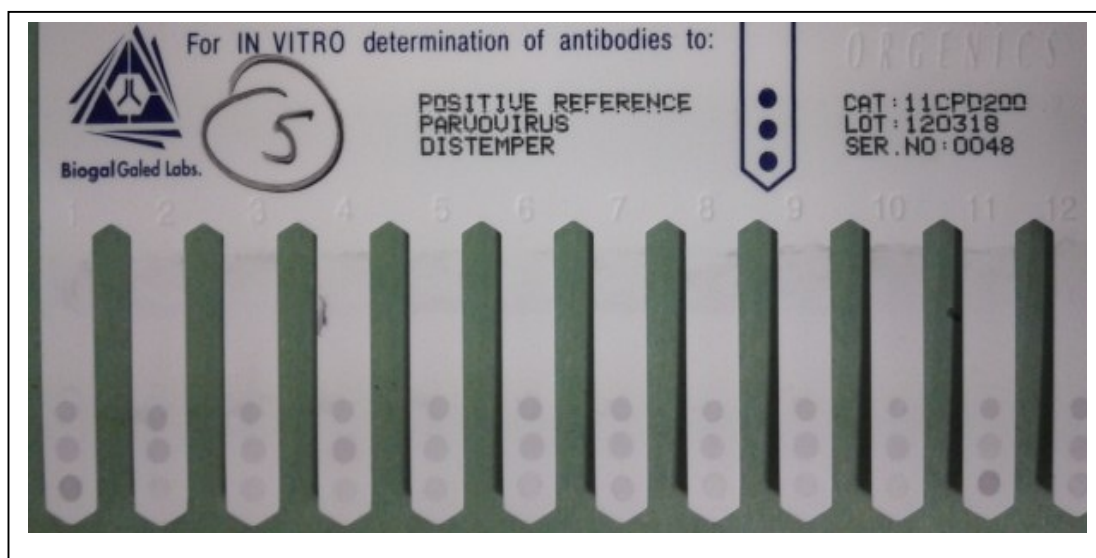


Figure 2.3: Biogal Laboratories, ImmunoComb®.

The first step of the test is to add 5ul of serum in a well in row A of the multi-compartment developing plate (diluting the samples in sample buffer 1 in 100). After adding the 12 samples the comb is inserted into the well containing sample and incubated for 5 minutes (samples

99

exposed to the antigen attached to the comb). For mixing during incubation, the comb is dipped up and down at the start of each incubation; this motion is repeated at least twice in all the remaining rows. Next, tweezers were used to pierce the foil of the next well (row B) as a wash step and the comb is inserted for 2 minutes; before transferring the comb from one well to the next, we pierced the foil of the next well and gently shook off excess liquid from the comb teeth onto a tissue. The comb will be in row C for 5 minutes (the secondary antibody was in those wells), after will be in row D & E for 2 minute in each well (washing step and fixation) and the last well (row F) for 5 minutes (substrate step). Upon completion of the colour development in row F, the comb is moved back to row E for 2 minutes for colour fixation and to stop the reaction. When the comb is out, it needs to be allowed to dry for 1-10 minutes. The test can be read manually with the comb scale or by an image developer matching the positive reference spot with Immune Response Status 3 or S3, which is the “cut-off” point. Anything ≥ 3 is positive meaning the titer is high enough to be protected against the virus, ≤ 3 mean inadequate immunity (low level of antibodies), ≥ 5 is high Positive which mean that you may have a recent infection or exposure to the virus.

2.7. Data Analysis

Statistical analysis was performed using SPSS version 13.0. Differences between mean score were analysed for significance by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Significant differences between groups were assessed using general linear model ANOVA followed by Turkey's post hoc test (for equal variances) or Games-Howell post hoc test (for unequal variances). Differences between cases and controls groups were analysed by independent-samples t test. The used of General Linear Model was justified by the need of determining whether the means of two groups differ (with or without SQSTM1/p62 mutations) procedure in which the calculations were performed using a least squares regression approach to describe the statistical relationship between some predictors and a continuous response variable. On GLM we choose to use a binary coding scheme (0, 1).

In general, variable transformation was used or apply to the data which didn't follow a normal distribution, as the viral titer (chapter 5), where the immune status ration were transform on tertiles. Similar approach was used on chapter 4, for the risk allele score.

On chapter 3 the Student's t test was used to look at the differences between two groups (with *SQSTM1* mutations or without) and Chi Square was used to compares observed frequencies to expected frequencies all in categorical data. Univariate and Multivariate regression analysis was used to study the association of the mutations with the composite severity score, described on more detail in the corresponding chapter. In this case multivariate linear regression was used as an approach for modeling the relationship between a scalar dependent variable (*severity*) and some explanatory variables (age of diagnosis, family history, mutations, calcium intake, occupation, previous musculoskeletal injury). In linear regression, data are modeled using linear predictor functions, and unknown model parameters are estimated from the data. The goal in this case was prediction, or forecasting. For the regression analysis the Normality was tested to determine if the data set was well modelled by a normal distribution and an informal approach to testing the normality was used by comparing a histogram of the sample to a normal probability curve, also the normal probability plot was used, a quantile-quantile plot (QQ plot) of the standardized

data against the standard normal distribution. For multivariate regression, adding and subtracting variables to the model one at a time and testing them individually or in small groups with t test allow us to decide on appropriate variable inclusion.

For the regression analysis the Normality was tested to determine if the data set was well modelled by a normal distribution. Comparing histogram of the sample to a normal probability curve, was an informal approach used on this case as well as normal probability plot where the standardized data against the standard normal distribution was tested.

Software was used for the data analysis (Minitab and SPSS). We use Regression to assess how independent predictors of disease are collectively associated with the severity Score. Given the potential for correlation among the predictors, we'll have Minitab display the variance inflation factors (VIF), which indicate the extent to which multi-collinearity is present in a regression analysis. A VIF of 5 or greater indicates a reason to be concerned about multi-collinearity.

On chapter 4: The meta-analysis was performed by Dr. Omar Albagha. In this case meta-analysis was used for comparing, contrasting and combining results from different studies or cohorts, in the hope of getting results on a wider group or population, identifying patterns among study results, sources of disagreement among those results, or other interesting relationships that may come to light in the context of multiple studies. Data were synthesized across cohorts by the meta-analysis using Review Manager software, we use the results from 5 different cohorts to examine the relation between risk allele score and markers of disease severity in the study group as a whole. No heterogeneity was found. The total size on this study adding all the cases used for each study is $N=1940$; but more specific details of the cohorts are in the table 4.1.

As will be mention on chapter 4, on figure 4.1 case we used the fixed effects model. The figure is referring to Standard mean difference which is calculated using the means from tertile 1 and tertile 2 in panel A, tertile 1 and tertile 3 in panel B taking in account the weigh. Basically the

figure 4.1 is showing that it is not heterogeneity between the results or in other words, the results are similar between the cohorts. The total size on this study adding all the cases used for each study is N=1940; but more specific details of the cohorts are in the table 4.1. The Mantel-Haenszel method was used to calculate odds ratios and confidence intervals for categorical variables, and the inverse variance method was used to calculate standardized mean differences for continuous variables.

On the same chapter, for each individual in each cohort, we assigned a score of 0, 1, or 2 to genotypes at the different 7 loci, depending on whether subjects carried the wild-type allele or were heterozygous or homozygous for the allele that was associated with PDB. Also a cumulative allelic risk score was created as described on previous publication (Albagha et al. 2013). A cumulative risk allele score was constructed by adding the variants together and relating this to markers of disease severity, alone and in combination with SQSTM1 mutations. The use of tertiles is justified also by the need of summarizing the data. Tertiles split the data into three (approximately) equally sized groups. This approach is particularly useful because the data was not symmetrically distributed.

Analysis of variance (ANOVA) and general linear model ANOVA were used to evaluate the differences between the genotyped groups for continuous variables, and the chi-square test was used for categorical variables. Post hoc tests were run to confirm where the differences occurred between groups, used just because an overall significant difference in group means was observed.

Statistical analysis on chapter 5 is based on the study of the differences between the levels of antibodies in cases and controls, and the differences were assessed by analysis of variance when the data were normally distributed or by Mann-Whitney test when the data were not normally distributed. Differences in the proportion of PDB patients and controls that tested positive for antibodies were assessed by the chi-square test. The relation between viral antibody concentrations and markers of disease severity was assessed by analysis of variance after categorising patients into tertiles of low, medium and high antibody levels for each virus. Additional analyses were performed in which the relationship between genotype and disease severity were analysed in relation to circulating viral antibodies. This analysis employed a general linear model analysis of variance, entering genotype, tertiles of antiviral antibodies,

gender and age into the model with calculation of least square means for the categorical variables. The significance level was set at 0.008 to account for the fact that antibodies against six different viruses were tested.

All data are presented as means \pm standard error of means (SEM) unless stated otherwise. Values of p less than 0.05 were considered significant, except on chapter 5 where we explained on detail.

2.8. Ethics

This study was approved by the multicentre ethics committee and the local ethical committees of the participating centres. All patients gave written informed consent to being included in the study.

Chapter 3:

Mutations of Sequestosome 1 are Associated with Severity and Clinical Outcome in Paget Disease of Bone

ABSTRACT

Genetic factors play an important role in the pathogenesis of PDB and the most important predisposing gene is *SQSTM1* which is mutated in about 10% of patients. Here we investigated the relationship between *SQSTM1* mutation status, disease severity and clinical outcome in 737 patients who took part in a randomised study of two different management strategies for the disease. Mutations of *SQSTM1* were detected in 80/737 (10.9%) patients. Mutation carriers had an earlier age at diagnosis (59.4 ± 11.5 vs. 65.0 ± 10.4 years, $p < 0.0001$); a greater number of affected bones (3.2 ± 1.2 vs. 2.1 ± 1.2 , $p < 0.001$) and more commonly had required orthopaedic surgery (26.2% vs. 16.1%, $p = 0.024$) and bisphosphonate therapy (86.3% vs. 75.2%, $p = 0.01$) than those without mutations. Quality of life as assessed by the SF36 physical summary score was significantly reduced in carriers (34.0 ± 11.3 vs. 37.1 ± 11.4 ; $p = 0.036$) and during the study fractures were more common (12.5% vs. 5.3%, $p = 0.011$) although most of these occurred in unaffected bone. This study demonstrates that *SQSTM1* mutations are strongly associated with disease severity and complications of PDB.

3.1. Materials and Methods

All the work in this chapter, practical work, and design of the experiment, results, statistics and calculations was performed by the candidate. PCR, genotyping by chromatograms analysis all performed by the candidate. The sequencing of the PCR products was done by the MRC sequencing service and the results were analysed by the candidate.

3.1.1. Genotyping for SQSTM1 mutations (exon 7 and exon 8)

Genomic DNA was extracted from peripheral blood using standard procedures, as described previously. The PCR methodology used as described previously (Barille et al. 1995) and is also described in detail under Materials and Methods chapter 2.

3.1.2. Amplifying Exon 7 and Exon 8

To identify any mutation on exon 7, a 454bp fragment was generated using the following primers: Forward, 5'TTAAAGTCACGCTGGGAACCTGCT3'; Reverse, 5'AGGGCAGGATGCTCTAAAGGG3'. To identify any mutation on exon 8, a 421bp fragment was generated, including entirely the encoding region, using the following primers: Forward, 5'TCTGGGCAGGCTCGGACACT3'; Reverse, 5'CCCTAAATGGCTTCTTGCACCC3'.

Amplification of both fragments was performed in a MJ Research thermocycler, conditions described in detail under Materials and Methods.

The PCR products were sequenced using the same primers in both forward and reverse directions. The traces were analysed by Chromas-pro software and compared with the reference sequence (NC_000005.8, GI: 51511721, NCBI Entrez Gene, <http://www.ncbi.nlm.nih.gov/>).

3.1.3. Ethics

This study was approved by the multicentre ethics committee and the local ethical committees of the participating centres. All patients gave written informed consent to being included in the study.

3.1.4. Statistical analysis

Student's t test was used to evaluate differences between patients with and without *SQSTM1* mutations for continuous variables, and the chi-square test was used for categorical variables. Univariate and multivariate regression analyses were used to evaluate predictors of disease severity.

3.2. Results

Patients who consented to provide DNA samples for analysis (n=737) were about 1 year younger than those who declined or were not asked to provide samples (n=578). (Mean \pm SD = 73.2 ± 7.8 vs. 74.8 ± 7.9 ; $p < 0.001$). They had an earlier age at diagnosis by about 3 years (64.4 ± 10.6 vs. 67.5 ± 10.8 , $p < 0.001$), but their gender distribution was similar (46% male vs. 48% male; $p = 0.43$) and the number of affected sites was similar (2.22 ± 1.3 vs. 2.22 ± 1.4 ; $p = 0.92$).

Mutations of *SQSTM1* were identified in 80/737 (10.9%) of the patients studied as summarised in Table 3.1. The most common mutation was P392L but several other mutations were found, including 7 novel mutations (Fig.3.1 and Fig.3.2); T1311G, causing an amino acid change from isoleucine to serine at codon 424 (I424S); C1238T, causing a premature stop codon at position 400 in place of a glutamine residue (Q400X) and an insertion in position 1348 of an C, which produced a frameshift with 48 extra aminoacids; an insertion of an G in position 1254, causing a premature stop codon at position 408 (D408X); a missense C1214T, which produce an aminoacid change from proline to serine at codon 392 (P392S); a missense mutation A1331G, causing an aminoacid change at codon 431, an isoleucine to valine (I431V); T1290C, causing an aminoacid change from leucine to proline at codon 417 (L417P); C1208G, causing an aminoacid change at codon 390 (A390G) an alanine to a glycine. All mutations were present in heterozygous form, with the exception of one patient who was a compound heterozygote for G425R and I424S.

Table 3.1: Prevalence and type of *SQSTM1* mutations in PRISM study

Nucleotide	AminoAcid Change	Mutation Type	Number of Patients	(%)
C1215T	P392L	Missense	60	75
1225insT	E396X	Truncating	2	2.5
C1238T	Q400X	Truncating	1	1.3
T1251C	M404T	Missense	1	1.3
A1250G	M404V	Missense	4	5
G1271A	G411S	Missense	1	1.3
T1311G	I424S	Missense	5	6.3
G1313A	G425R	Missense	5	6.3
G1313A/T1311G	G425R/I424S	Missense	1	1.3
TOTAL			80	100

The data of this table is obtained from 737 Prism cases analysed

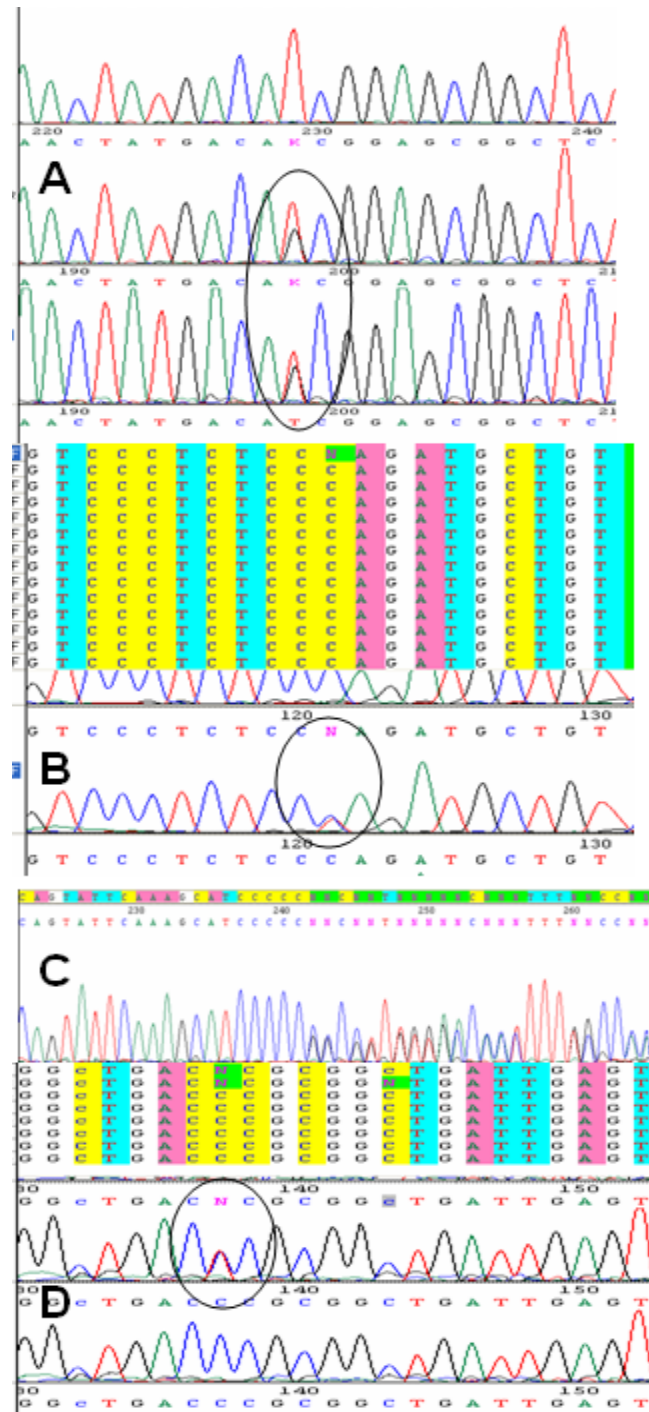


Figure 3.1: Some of the new mutations. A: Missense, T1311G, to produce an amino acid change I424S. B: truncating, replacement of a C1238T, producing a truncated protein Q400X. C: insertion of a C in position 1348, producing a frame shift with extra 48aa. D: missense, C1214T, producing an amino acid change P392S.

The clinical characteristics of patients with *SQSTM1* mutations differed significantly from those without mutations as summarised in Table 3.2.

Table 3.2. Relation between *SQSTM1* Mutation Status and Clinical Features of PDB at Baseline

Variable	<i>SQSTM1</i> +ve n=80	<i>SQSTM1</i> -ve n=657	<i>p</i> Value
Male	(47.5%) 38) 71.	(54.2%) 356) 73.	.13
Age at recruitment	1 ± 8.1 1.2	4 ± 7.8 1.4	.19
Alkaline phosphatase	5 ± 1.02	5 ± 1.51	.12
Previous courses of Bisphosphonate			
0	(13.7%) 11)	(24.8%) 163)	
1	(35%) 28)	(40.1%) 269)	.01
2	(30%) 24)	(22.8%) 150)	
3 or more	(21.2%) 17) 59.	(11.4%) 75)	
Age at diagnosis	4 ± 11.5	65 ± 10.4	<.0001
Family history of PDB	(42.5%) 34)	(11.3%) 74)	<.001
Number of bones affected	3.2 ± 2	2.1 ± 1.3	<.0001
Patients with bone deformity	(42.5%) 34)	(35.7%) 234)	.23
Any fracture	(38.7%) 31)	(40.3%) 265)	.78

Fracture in unaffected bone	20 (25%)	200 (30.4%)	.31
fracture in Pagetic bone	11 (13.7%)	65 (9.8%)	.28
Orthopaedic surgery	21 (26.2%)	106 (16.1%)	.024
Skull disease and hearing aid	8 (10%)	46 (7%)	.33
SF-36 bodily pain	38.6 ± 11	40.4 ± 11.4	.18
SF-36 physical summary score	49.34 ± 11.3	49.1 ± 11.4	.036
SF-36 mental summary score	2 ± 11.7	4 ± 11.5	.90
Composite disease severity score	7.9 ± 3.3	6 ± 2.6	<.0001

Values are mean SD or number (%). The p values refer to the differences between the genotype groups assessed by Student's test or chi-square test. The ALP values have been standardized to the upper limit of the reference range, which was set at 1.0.

There was no difference between the genotype groups in terms of age, gender or total ALP at baseline although it should be noted that a high proportion of patients had previously been treated with bisphosphonates. Patients with *SQSTM1* mutations had an earlier age at first diagnosis than those without mutations (59.4 ± 11.5 vs. 65.0 ± 10.4 ; $p < 0.0001$), had previously required more courses of bisphosphonate therapy for PDB (86.3% vs. 75.2%; $p = 0.01$), had a greater number of affected bones ($p < 0.0001$) and had required orthopaedic surgery more frequently (26.2% vs. 16.1%; $p = 0.024$). Several other complications of the disease tended to be more common in carriers of *SQSTM1* mutations including bone deformity, previous fractures through affected bone and patients with skull disease who used a hearing aid for deafness but the difference between the groups was not significant for these variables (Fig.3.1). Overall disease severity, as assessed by the composite score described in the methods section was significantly greater in *SQSTM1* mutation carriers (7.9 ± 3.3 vs. 6.0 ± 2.6 ; $p < 0.0001$). In keeping with this the SF36 physical summary score was lower in *SQSTM1* mutation carriers

(34.0 ± 11.3 vs. 37.1 ± 11.4 ; $p=0.036$), although there was no difference in SF36 bodily pain score or SF36 mental summary score between *SQSTM1* mutation carriers and non-carriers.

The relationship between *SQSTM1* mutation status and response to treatment is shown in Table 3.3.

Table3.3. Relation between *SQSTM1* Mutation Status and Response to treatment

Variable	<i>SQSTM1</i> +ve (n=80)	<i>SQSTM1</i> -ve (n=637)	p Value
Intensive treatment	38 (47.5%)	338 (51.5%)	0.5
Any fracture	10 (12.5%)	35 (5.3%)	0.011
Fracture in unaffected bone	10 (12.5%)	26 (4.0%)	0.001
Fracture in affected bone	0 (0%)	10 (1.8%)	0.25
Orthopaedic surgery	8 (10.0%)	39 (5.9%)	0.16
Bisphosphonate therapy	49 (61.2%)	402 (61.0%)	0.97
Bisphosphonate dose (mg)*			
Alendronate	259±1462	106±802	0.15
Etidronate	2610±21488	1046±8207	0.2
Pamidronate	46.9±118	53.5±176	0.74
Risedronate	2013±3253	2113±3436	0.79
Tiludronate	6640±27129	6283±23065	0.86
Alkaline phosphatase, 24 months	1.01±0.70	0.93±0.71	0.34
SF-36 bodily pain, 24 months	38.0±9.8	41.0±11.0	0.04
SF-36 physical summary, 24 months	32.7±9.4	36.8±11.3	0.007
SF-36 mental summary, 24 months	47.2±12.5	48.1±11.2	0.55

Values are mean SD or number (%). The p values refer to the differences between the genotype groups assessed by Student's t test, chi-square test, or (in the case of bisphosphonate dose) Kruskal-Wallis test. The ALP values have been standardized to the upper limit of the reference range, which was set at 1.0.

***Total dose of bisphosphonate given during the study, in milligrams.**

The proportion of patients allocated to intensive and symptomatic treatment was similar in the two genotype groups and there was no difference between the groups in the doses of bisphosphonates administered, the number of patients who required orthopaedic surgery or the level of total ALP at 24 months. Fractures were significantly more common in patients with *SQSTM1* mutations (12.5% vs. 5.3%; p=0.011) but these predominantly occurred in non-

Pagetic bone. The SF36 physical summary score at 24 months was lower in *SQSTM1* mutation carriers as compared with non-carriers (32.7 ± 9.4 vs. 36.8 ± 11.3 ; $p=0.007$). Bodily pain as assessed by SF36 was also significantly worse in *SQSTM1* mutation carriers at 24 months (38.0 ± 9.8 vs. 41.0 ± 11.0 ; $p=0.04$) but there was no significant difference in the SF36 mental summary score between the groups.

In order to identify the predictors of a poor clinical outcome in PDB we used univariate and multivariate regression analysis to identify factors that were associated with the composite disease severity score at baseline. Univariate analysis showed that age ($p=0.01$), age at diagnosis ($p<0.001$), family history ($p<0.001$), and the presence of a *SQSTM1* mutations ($P<0.001$) were all significant predictors of disease severity score (Fig.3.2). None of the environmental variables studied including dietary calcium intake, occupation, or previous musculoskeletal injury predicted disease severity score (all p values > 0.4). Multivariate analysis (Table 3. 4) identified four independent predictors of disease severity score which together accounted for 29.1% of the variance in disease severity score. These were *SQSTM1* mutation; age at diagnosis, age at the baseline visit, and a positive family history of PDB (Fig3.2).

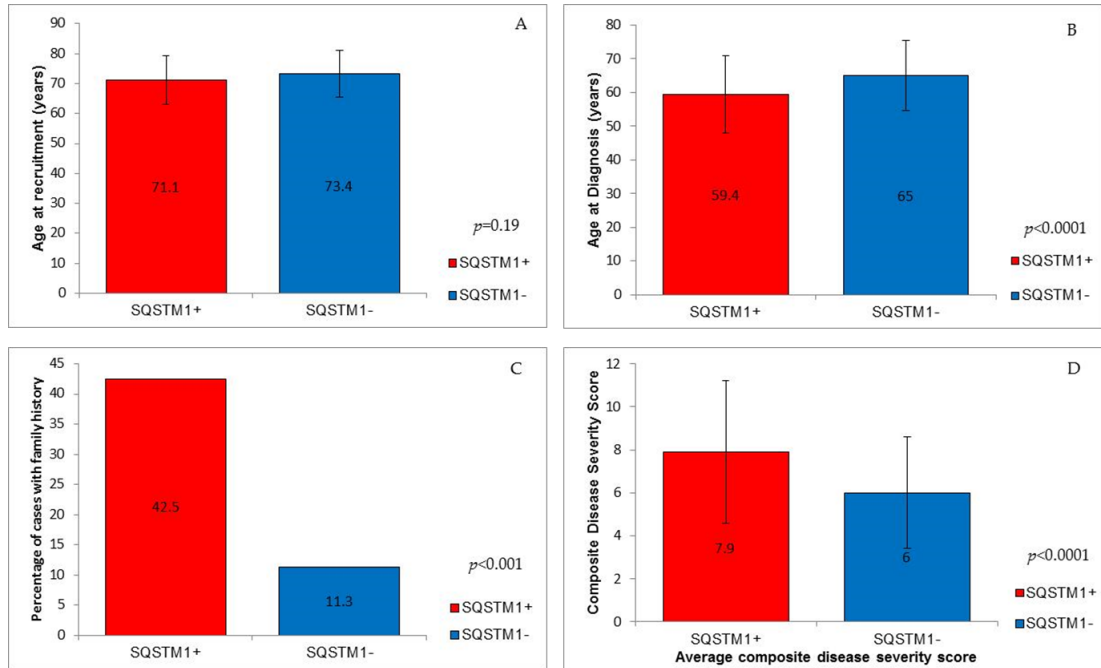


Figure 3.2: *SQSTM1* Mutation status in relation with age at recruitment, age at diagnosis, family history and composite disease severity score. Panel A showing relation between the presence of the *SQSTM1* mutations and the age of recruitment. Panel B showing the relation between the age at diagnosis and the occurrence of the *SQSTM1* mutations. The error bars are mean \pm SD. In the panel C, represent the percentage of cases with family history, the values are expressed on percentage (%). The panel D represent the relation between the severity score and the occurrence of *SQSTM1* mutations. The p values refer to the differences between the genotype groups assessed by Student's test or chi-square test.

Table 3.4: Independent Predictors of Baseline Disease Severity Score in the PRISM Study.

Predictor	Coefficient	SD	T	p Value
Constant	9.746	0.888	10.97	<.0001
<i>SQSTM1</i> mutation	0.863	0.285	3.03	.003
Family history	0.862	0.249	3.47	.001
Age at diagnosis	-0.160	0.010	-15.60	<.0001
Age at baseline	0.112	0.013	8.29	<.000

Regression equation severity=9.746+0.863 (mutation) +0.863 (family history)-0.160 (age at diagnosis) +0.113 (age at baseline). S=2.28R²=30.3%R² (adj) =29.1%.

3.3. Discussion

This study has shown that *SQSTM1* mutations are an important marker of disease severity and complications in PDB patients attending secondary referral centres in the UK. In keeping with previous smaller studies (Collet et al. 2007; Hocking et al. 2004) we found that patients who carried *SQSTM1* mutations had a younger age at diagnosis and more extensive disease than those without mutations. We have now shown for the first time that *SQSTM1* mutation carriers are at greater risk of developing disease complications and have greater impairment of quality of life than non-carriers. It is interesting to note however that the reduction in quality of life was restricted to the physical summary score of SF36, which were between 12 and 16 points below the population normal values of 50 whereas the SF36 mental summary scores were completely normal in both screened groups. This suggests that whilst PDB adversely affects physical functioning, those patients who are affected seem to be able to overcome adversity and cope with their disability.

Analysis of the relationship with treatment response showed no significant difference between *SQSTM1* mutation carriers and non-carriers with regard to the biochemical response to bisphosphonate therapy. The amounts of bisphosphonate administered during the study were equivalent in both genotype groups; ALP levels were suppressed to an equal extent and the

requirement for orthopaedic surgery was similar. Quality of life remained lower in *SQSTM1* mutation carriers than non-carriers after 24 months of treatment, however, suggesting that once complications of PDB and physical impairment have developed, they are not influenced substantially by therapeutic intervention. This is in keeping with the results reported for the PRISM study as a whole where we found that neither symptomatic management nor intensive management had a significant impact on quality of life or disease complications (Langston et al. 2007a). We were interested to note that fractures were significantly more frequent in patients with *SQSTM1* mutations during the study although these predominantly occurred through unaffected bone. Previous studies have shown that fracture risk is increased in PDB (Melton, III et al. 2000b; van Staa et al. 2002a), and in the PRISM study we found that about 80% of these fractures are in unaffected bone.

Whilst the increased risk of fractures in Pagetic bone is easily explained on the basis of reduced bone quality, the reason for the increased risk in unaffected bone is less clear. Previous studies have shown that PDB is associated with an increased risk of osteoarthritis (Helliwell 1995; Melton, III et al. 2000a; van Staa et al. 2002b). This condition is known to be associated with an increased risk of fracture (Arden et al. 2006). Another potential reason for an increased fracture risk of unaffected bone in PDB would be deformity with resulting postural instability. This would be in keeping with the trend for patients with *SQSTM1* mutations to have worse bone deformity than those without mutations.

Epidemiological studies have suggested that low dietary calcium intake during childhood is associated with an increased risk of developing PDB (Siris 1994). In this study we observed no association between disease severity and consumption of dairy products during childhood or at other points in life. Repetitive mechanical loading has also been suggested to play a role in targeting of Paget's disease to specific bones in the skeleton although this is mainly based on anecdote (Solomon 1979). Based upon this observation, we looked at the relationship between occupation and disease severity, but no significant association was observed, nor did we observe an association between previous musculoskeletal injury and severity of PDB.

A limitation of our study is that the conclusions only hold true for participants in whom DNA samples were obtained. We feel that the results are likely to be fairly representative of PDB

patients being treated in a secondary care setting however, since the characteristics of subjects who provided DNA samples were broadly similar to those who did not.

Previous studies have shown that intervention with potent bisphosphonates such as Zoledronic acid and Risedronate are very effective at suppressing bone turnover in PDB (Reid et al. 2005; Siris et al. 1998). The effects of these agents on quality of life and disease complications are modest, however, probably because many patients have already developed irreversible skeletal damage by the time treatment is initiated. Our data raise the possibility that genetic testing for *SQSTM1* mutations in patients with a family history of the disease might be warranted so that carriers of these mutations can be kept under close surveillance for early signs of the disease developing.

It is important to emphasise that if a programme of genetic testing was to be implemented, then properly designed studies would need to be conducted to examine whether prophylactic bisphosphonate therapy would be of clinical benefit in asymptomatic *SQSTM1* gene carriers. In this regard it is relevant to point out that the current generation of *SQSTM1* gene carriers seems to have a delayed onset of PDB as compared with their parents (Bolland et al. 2007). Accordingly, the timing of any intervention would need to be carefully chosen to take the likely age at onset into account. Other issues such as anxiety associated with having a genetic test and cost-effectiveness would also need to be addressed to determine if the potential clinical benefit of genetic testing and intervention outweighs the costs of mutation screening and drug treatment. A Trial is now in progress, called the ZiPP study (Zoledronate in the Prevention of Paget's disease; ISRCTN11616770) in which we are addressing these issues. The ZiPP study aims to explore the risk and benefits of prophylactic zoledronic acid therapy versus placebo in asymptomatic patients with *SQSTM1* mutations who have not yet been diagnosed with PDB. The primary aim of the ZiPP study is to determine whether bisphosphonate therapy can prevent the development of elevated bone turnover and bone lesions in mutation carriers but information is also being gathered on clinically relevant outcomes such as pain, quality of life, and anxiety and depression. At present, we do not feel that *SQSTM1* Mutation screening is indicated in routine clinical practice but it will be important to re-evaluate this in the light of the results of the ZiPP study, which should be available in 5 to 7 years' time.

Chapter 4:

Identification of Novel Genetic Markers that predict Disease severity and Complications in Paget's disease of Bone

ABSTRACT

PDB is a common skeletal disorder affecting about 2% of people over the age of 55 in the UK. Some patients develop progressive disease complicated by deafness, bone deformity, fractures and osteoarthritis. Mutations of *SQSTM1* are found in about 10% of PDB patients and are significantly associated with disease severity (Visconti et al. 2010). Recently, seven additional susceptibility loci for PDB have been identified by GWAS in patients without *SQSTM1* mutations. The aim of this study was to determine if these novel loci are also associated with clinical severity and extent of PDB, either alone or in combination with *SQSTM1* mutations.

Genotyping was conducted for the seven PDB susceptibility SNPs (rs10494112, rs4294134, rs2458413, rs1561570, rs10498635, rs5742915, rs3018362) and previous results of genotyping for *SQSTM1* was used. The samples used were 770 participants of the PRISM study and in a replication sample of 1170 PDB patients from centres in Italy, Western Australia and Spain. For statistical analysis, the patients were divided into tertiles based on the cumulative number of risk allele carried and the association between carriage of the alleles, extent of PDB, disease complications and a composite disease severity score (previously mentioned) was analysed in relation to *SQSTM1* mutation status.

4.1. Materials and Methods

In this chapter, the primary analysis for PRISM samples was done by the candidate. The sequencing of the PCR products was done by the MRC sequencing service. Genotyping for the other SNPs that attained genome wide significance for association with PDB (rs10494112, rs4294134, rs2458413, rs1561570, rs10498635, rs5742915, rs3018362) **was performed externally at the genetics core of the Wellcome Trust Clinical Research Facility.** Genotypes for cases and controls were called using BeadStudio v3.2 (Illumina, Inc.) by following the manufacturer's recommended protocol and the meta-analysis **was performed by Dr. Omar Albagha.**

4.1.1. Patients

As mentioned on the materials and methods chapter, the primary analysis was conducted in participants of the Paget's

Disease, Randomised Trial of Intensive versus Symptomatic Management (PRISM) study (ISRCTN12989577) which was a randomised comparative trial of two treatment strategies for PDB (Langston et al. 2007c). The present study is based on a subgroup of 770 study participants who consented to provide a blood sample for genetic analysis. For replication, four separate clinic based cohorts of PDB patients were used. The GenePage cohort, comprising 384 unrelated Italian patients with Paget's disease recruited from 13 Italian centres as previously described (Falchetti et al. 2009); the Naples/Siena cohort comprised a non-overlapping cohort of 363 unrelated Italian patients with Paget's disease recruited from northern, central and southern Italy as described (Gennari et al. 2010); the Salamanca cohort comprising 191 unrelated patients attending a specialist clinic for Paget's disease in Salamanca, Spain (Corral-Gudino et al. 2012); and the Western Australian cohort comprising 232 unrelated patients attending a specialist clinic in Perth (Rea et al. 2009).

4.1.2. Disease Extent and Clinical Assessment

In all cohorts, disease extent was assessed on the basis of radionuclide bone scan by counting the number of affected sites as explained previously. Clinical assessment, have been also explained on the Materials and Methods chapter.

4.1.3. Genotyping

Genotyping for *SQSTM1* has been described in detail on the Materials and Methods chapter. Genotyping for the other SNPs that attained genome wide significance for association with PDB (rs10494112, rs4294134, rs2458413, rs1561570, rs10498635, rs5742915, rs3018362) **was performed externally at the genetics core of the Wellcome Trust Clinical Research Facility** using either using the Illumina HumanHap300-Duo BeadChip v2 array or the Sequenom MassARRAY iPLEX platform (Albagha et al. 2011). Genotypes for cases and controls were called using BeadStudio v3.2 (Illumina, Inc.) by following the manufacturer's recommended protocol. **All the analysis was performed by Dr. Omar Albagha** using BeadStudio and quality-control metrics such as cluster separation, AB T mean (the mean normalized theta values of the heterozygote cluster) and AB R mean (the mean normalized intensity of the heterozygote cluster) to exclude badly performing SNPs. Samples with a call rate of less than 90% were excluded (n=30). A number of randomly selected samples were genotyped on the different genotyping platforms (n=96 on Illumina and Sequenom; n=12 on all three platforms) and the cross-platform genotype concordance rate was >99.9%. The data were then subjected to further quality control measures using PLINK (Purcell et al. 2007).

4.1.4. Ethics

The study was approved by the multicentre ethics committee and the local ethical committees of the participating centres. All patients gave written consent to be included in the study.

4.1.5. Statistical analysis

The meta-analysis was performed by Dr. Omar Albagha. In this case meta-analysis was used for comparing, contrasting and combining results from different studies or cohorts, in the hope of getting results on a wider group or population, identifying patterns among study results, sources of disagreement among those results, or other interesting relationships that may come to light in the context of multiple studies.

Data were synthesized across cohorts by the meta-analysis using Review Manager software, we use the results from 5 different cohorts to examine the relation between risk allele score and markers of disease severity in the study group as a whole. No heterogeneity was found. The total size on this study adding all the cases used for each study is N=1940; but more specific details of the cohorts are in the table 4.1.

As mention on figure 4.1 case we used the fixed effects model. The figure is referring to Standard mean difference which is calculated using the means from tertile 1 and tertile 2 in panel A, tertile 1 and tertile 3 in panel B taking in account the weigh. Basically the figure 4.1 is showing that it is not heterogeneity between the results or in other words, the results are similar between the cohorts. The total size on this study adding all the cases used for each study is N=1940; but more specific details of the cohorts are in the table 4.1. The Mantel-Haenszel method was used to calculate odds ratios and confidence intervals for categorical variables, and the inverse variance method was used to calculate standardized mean differences for continuous variables.

For each individual in each cohort, we assigned a score of 0, 1 or 2 to genotypes at the rs10494112, rs4294134, rs2458413, rs1561570, rs10498635, rs5742915, and rs3018362 loci, depending on whether subjects were homozygous or were heterozygous or homozygous for the allele that was associated with PDB. The score was adjusted for each locus depending on the strength of the association with PDB derived from the odds ratio for association with PDB as described previously (Albagha et al. 2011). The adjustment factor for each allele carried

was 1.72 for rs10494112; 1.45 for rs4294134, 1.40 for rs2458413, 1.67 for rs1561570, 1.44 for rs10498635, 1.34 for rs5742915, and 1.45 for rs3018362.

The scores were then added across all seven loci to create a cumulative allelic risk score and divided subjects from each cohort into tertiles based on the cumulative risk score. The cumulative allelic risk score was created as described on previous publication (Albagha et al. 2013). A cumulative risk allele score was constructed by adding the variants together and relating this to markers of disease severity, alone and in combination with *SQSTM1* mutations. The use of tertiles is justified also by the need of summarizing the data. Tertiles split the data into three (approximately) equally sized groups. This approach is particularly useful because the data was not symmetrically distributed.

For *SQSTM1* mutations, a score of 0 or 1 was assigned depending on whether or not a mutation was present. We also devised a genetic risk score in which patients were classified into three groups based on their risk allele score and *SQSTM1* mutation status. Low risk patients were defined as those in the lowest two tertiles of risk allele score who were negative for *SQSTM1* mutations. Medium risk patients were defined as those in the highest tertile of risk allele score or those with *SQSTM1* mutations. High risk patients were defined as those in the top tertile of risk allele score and positive for *SQSTM1* mutation.

Analysis of variance (ANOVA) and general linear model ANOVA were used to evaluate differences between the genotype groups for continuous variables and the chi-square test was used for categorical variables. Data was synthesized across cohorts by meta-analysis using Review Manager software. The Mantel-Haenszel method was used to calculate odds ratios and confidence intervals for categorical variables and the inverse variance method was used to calculate standardised mean differences for continuous variables.

4.2. Results

4.2.1. Characteristics of study cohorts

The characteristics of the cohorts included in the study are summarised in Table 4.1. The average age at recruitment ranged from 67 to 75 years and the average age at diagnosis from 58 to 68 years. There was a predominance of male subjects in each cohort ranging from 53% to 60% and between 8.6% and 19.8% of patients reported a family history of the disease. The frequency of complications varied between cohorts. Fractures through Pagetic bone ranged from 3.1% in Salamanca to 12.7% in the GenePage cohort whereas the prevalence of bone deformity ranged from 0.5% in the GenePage cohort to 59.7% in the Salamanca cohort. The frequency of deafness with skull involvement ranged from 5.9% in the GenePage cohort to 17.3% in the Naples/Siena cohort and the frequency with which orthopaedic surgery had been required for PDB ranged from 6.1% in the Naples/Siena cohort to 16.9% in the PRISM cohort. The frequency with which patients had previously been given bisphosphonates therapy for Paget's disease: ranged from 57.1% in the Salamanca cohort to 85.6% in the GenePage cohort.

Table 4.1: Characteristics of Included studies.

	PRISM	Naples/ Siena	GenePage	Salamanca	Perth
Number	770	363	384	191	232
<i>SQSTM1</i> +ve	82 (10.6%)	37 (10.2%)	30 (7.8%)	14 (7.3%)	17 (7.3%)
Age (9years)	73.0 ± 8.0	67.4 ± 11.5	75.8 ± 10.0	74.4 ± 9.9	72.9 ± 9.9
Age at diagnosis	64.3 ± 10.6	58.6 ± 11.7	62.4 ± 10.3	68.0 ± 11.1	62.2 ± 11.6
Male	407 (52.9%)	210 (57.8%)	206 (53.6%)	114 (59.8%)	133 (57.3%)
Family History	113 (14.6%)	72 (19.8%)	33 (8.6%)	28 (14.6%)	46 (19.8%)
Affected bones	1.8 ± 1.05	2.4 ± 2.02	1.5 ± 0.91	2.8 ± 2.0	2.6 ± 2.2
Disease severity	5.1 ± 2.5	7.1 ± 3.11	5.7 ± 1.52	6.6 ± 3.3	6.4 ± 2.8
Paget Fracture	81 (10.5%)	29 (7.9%)	49 (12.7%)	6 (3.1%)	15 (6.4%)
Bone deformity	280 (36.3%)	162 (44.6%)	2 (0.5%)	114 (59.7%)	N/A
Deafness	57 (7.5%)	63 (17.3%)	23 (5.9%)	25 (13.1%)	N/A
Previous Bisphosphonate s	577 (75.0%)	225 (62.0%)	329 (85.6%)	109 (57.1%)	194 (83.6%)
Orthopaedic Surgery	130 (16.9%)	22 (6.1%)	38 (9.8%)	23 (12.0%)	N/A

The first row is the total number of cases used in each study. The percentage are calculated according to the number of cases in each cohort. Values are mean ± SD or number (%).

4.2.2. Association between PDB susceptibility alleles and severity in the PRISM study

The association between PDB susceptibility alleles, disease extent and complications in the PRISM cohort are shown in Table.4.2. There was a significant association between risk allele score and number of affected bones with evidence of an allele dose effect ($p=0.04$). A similar trend was observed for disease severity score, but the differences were not significant. Family history of PDB was significantly associated with risk allele score in this subgroup of patients ($p=0.01$) as was the number of previous courses of bisphosphonates received for PDB ($p=0.04$). There was no significant association between allele risk score and sex, age, age at diagnosis of PDB, quality of life measures, the presence of deafness owing to PDB (reflected by use of a hearing aid and skull involvement), fractures through Pagetic bone, orthopaedic surgery, or bone deformity.

Table 4.2.: Susceptibility alleles and severity of PDB in PRISM study at baseline visit.

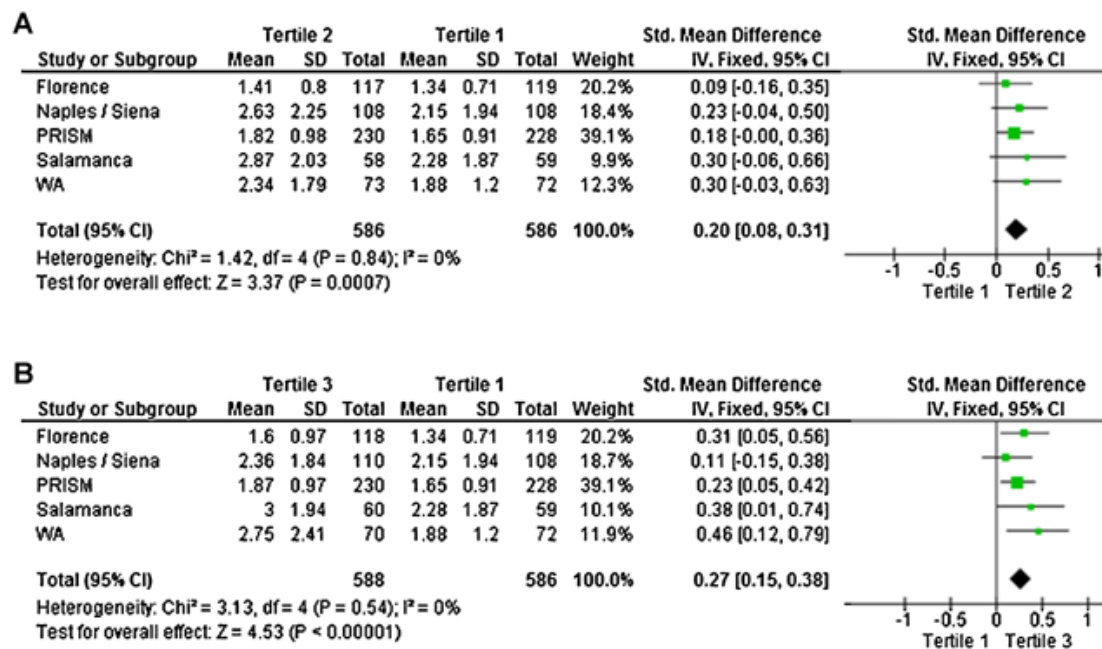
Variable		Tertile 1 (n=228)	Tertile 2 (n=230)	Tertile 3 (n=230)	p-value
Age (years)		73.3 ± 7.8	73.7 ± 8.1	72.7 ± 7.8	0.47
Male		121 (53.1%)	131 (57.0%)	117 (50.9%)	0.39
Age at diagnosis		65.2 ± 10.4	65.7 ± 10.4	64.0 ± 10.5	0.19
Family History of PDB		18 (7.9%)	23 (10.0%)	38 (16.5%)	0.01
Patients with Bone deformity		84 (36.8%)	75 (32.6%)	90 (39.1%)	0.33
Fracture in Pagetic Bone		17 (7.5%)	29 (12.6%)	25 (10.9%)	0.18
Orthopaedic surgery for PDB		36 (15.8%)	38 (16.5%)	37 (16.1%)	0.97
Deafness and skull PDB		17 (7.5%)	17 (7.4%)	14 (6.1%)	0.80
Number of affected bones		1.66 ± 0.92	1.82 ± 0.98	1.87 ± 0.98	0.04
Disease severity score		4.82 ± 2.18	4.87 ± 2.34	5.19 ± 2.22	0.17
Previous bisphosphonates	0	69 (30.3%)	68 (29.6%)	41 (17.7%)	0.04
	1	87 (38.2%)	91 (39.6%)	98 (42.6%)	
	2	47 (20.6%)	48 (20.9%)	57 (24.8%)	
	3 or more	25 (10.9%)	23 (10.0%)	34 (14.7%)	
Pain and quality of life	SF36 bodily pain	40.6 ± 10.6	40.5 ± 10.6	40.7 ± 11.1	0.98
	SF36 physical summary	37.7 ± 11.0	37.0 ± 11.6	37.1 ± 11.8	0.77
	SF36 mental summary	49.4 ± 10.7	49.5 ± 11.9	49.7 ± 11.5	0.94
	Alkaline Phosphatase	1.18 ± 0.85	1.37 ± 1.25	1.21 ± 0.91	0.10

Values are mean ± SD or number (%). The p-values refer to the differences between the genotype groups assessed by ANOVA or Chi-square test. The ALP values have been standardised to the upper limit of the reference range which was set at 1.0

4.2.3. Meta-analysis of PDB susceptibility alleles in relation to disease severity

In order to determine if risk alleles were associated with disease severity in other populations we employed meta-analysis to examine the relation between risk allele score and markers of disease severity in the study group as a whole. There was a highly significant association between risk allele score and both disease extent and severity with evidence of an allele dose effect. The number of affected bones was significantly greater in tertile 2 compare with tertile 1 (standardized mean difference 0.20 [0.08-0.31], $p=0.0007$) and greater still in tertile 3 compared with tertile 1 (standardized mean difference 0.27 [0.15-0.38], $p=0.00001$) (Fig. 4.1).

Fig. 4.1: Risk Allele Score and Number of affected bones.



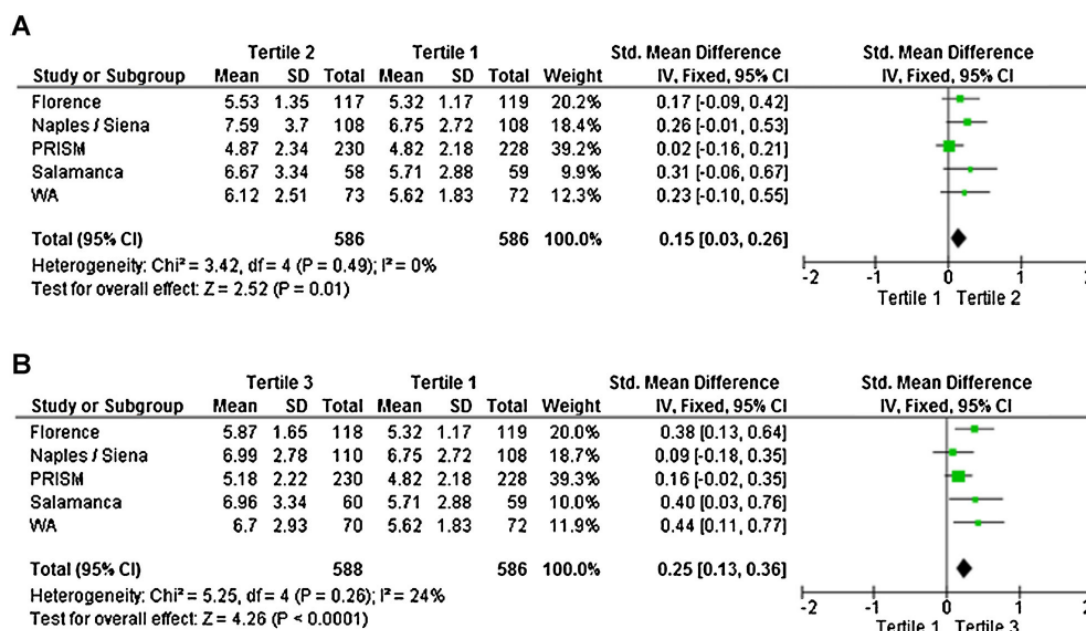
Meta-analysis of association between Risk allele score and number of affected bones on pagetic SQSTM1-mutation-negative individuals, using a fixed effects model, comparing tertile 1 with tertile 2 (panel A) and tertile 1 with tertile 3 (panel B).

A similar effect was noted for disease severity score, which was greater in tertile 2 than in tertile 1 (0.15 [0.03-0.26], $p=0.01$) and greater still in tertile 3 compared with tertile 1 (standardised mean difference 0.25 [0.13-0.36], $p<0.0001$) (Fig. 4.2). There was no significant

association between fractures through Pagetic bone, use of a hearing aid and Paget's disease of the skull, orthopaedic surgery for Paget's disease, or bone deformity and risk allele score. However, the number of previous courses of bisphosphonates received for was also significantly greater for those within tertile 3 as opposed to tertile 1 (0.22 [0.10-0.33], $p=0.0002$) (Fig. 4.3).

Analysis of individual loci for PDB susceptibility in relation to disease extent and severity in the whole cohort of SQSTM1 negative subjects, revealed weakly positive associations for some markers. These included rs104941, rs2458413, rs1561570, and rs3018362 with the number of affected bones ($p=0.048$, $p=0.012$ and $p=0.030$ and $p=0.043$ respectively); and rs2458413 for disease severity ($p=0.041$).

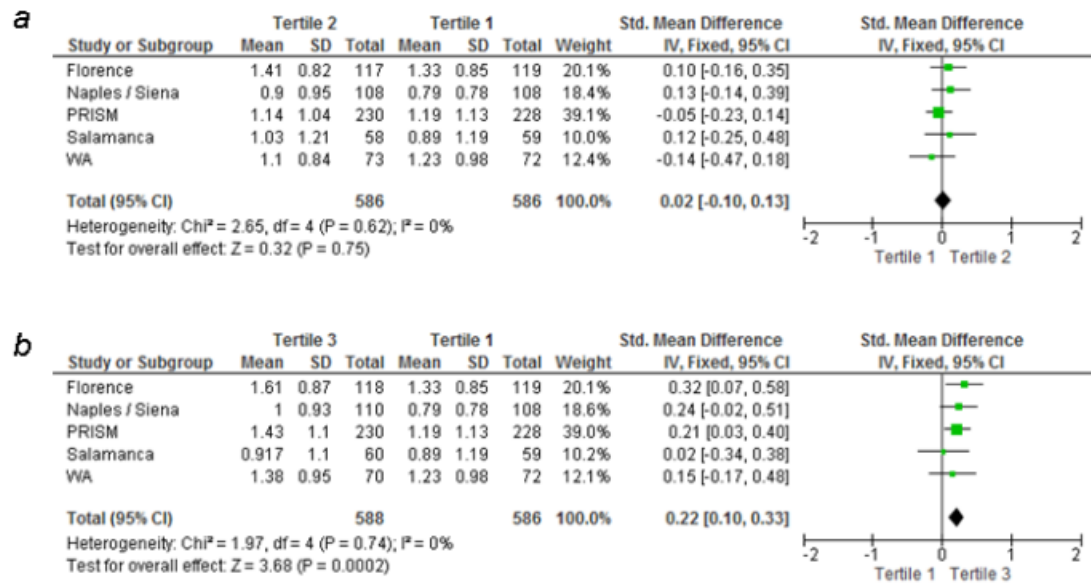
Fig. 4.2: Risk allele score and disease severity score.



Meta-analysis of association between risk alleles and severity score on pagetic

SQSTM1-mutation negative individuals, using a fixed effects model, comparing tertile 1 with tertile 2 (panel A) and tertile 1 with tertile 3 (panel B).

Fig. 4.3: Risk allele score and previous courses of bisphosphonates.



Meta-analysis of association between risk alleles and previous courses of bisphosphonates on pagetic *SQSTM1*-mutation negative individuals, using a fixed effects model, comparing tertile 1 with tertile 2 (panel a) and tertile 1 with tertile 3 (panel b).

4.2.4. Interaction between *SQSTM1* mutations, risk alleles and disease severity.

Since we and others have previously reported that *SQSTM1* mutations influence severity of PDB (Collet et al. 2007; Hocking et al. 2004; Visconti et al. 2010) we looked for evidence of an interaction between risk allele score and *SQSTM1* mutations in predicting disease severity. We analysed the relationship between risk allele score (lowest two tertiles versus highest tertile), *SQSTM1* mutations, number of affected bones and overall severity using general linear model ANOVA, also entering age, gender and study site into the model for the whole study population. The results are summarized in Table 4.3. For number of affected bones, male gender ($p < 0.0001$), risk allele category ($p < 0.0001$), *SQSTM1* mutations ($p < 0.0001$), and study center ($p < 0.0001$) were all significant predictors. For disease severity score, significant predictors were male gender ($p < 0.0001$), family history of Paget's ($p = 0.001$), *SQSTM1* mutations ($p < 0.0001$), risk allele category ($p < 0.0001$), center

($p = 0.002$ to 0.0001), and age ($p = 0.01$, beta coefficient 0.005). The effect size of *SQSTM1* mutations on number of affected bones and overall disease severity as reflected by the β -coefficients was about three times larger than that of the risk allele score.

Because both risk allele score and *SQSTM1* were independent predictors of disease extent and severity, we combined information from both markers to create a genetic risk score, dividing patients into three groups: group 1 (*SQSTM1* negative and tertile 1 of risk allele score); group 2 (*SQSTM1* negative and tertiles 2 and 3 of risk allele score); and group 3 (*SQSTM1* positive and tertiles 1 to 3 of risk allele score).

Analysis of various markers of disease severity in the whole study population showed a highly significant and stepwise increase in disease extent, severity, and number of previous bisphosphonates given for Paget's in relation to the genetic risk score (Table 4.4). When we defined "severe disease" as a disease severity score of 7 or greater (representing the top 20% of the whole population), there was a stepwise increase in severity according to genetic risk score. In terms of specificity and sensitivity, the highest category of genetic risk score had 70% specificity and 55% sensitivity for predicting severe disease.

Table 4.3.: Predictors of Disease Severity and Extent in the Whole Study Population.

	No. of affected bones			Disease Severity score		
	Mean±SD	Beta	<i>p</i> Value	Mean±SD	β	<i>p</i> Value
Gender						
Female	2.67±0.07	-0.137	<0.0001	6.88±0.12	-0.199	<0.0001
Male	2.95±0.07	—		7.28±0.12	—	
Family history						
No	2.72±0.06	-0.092	0.055	6.80±0.11	-0.277	0.001
Yes	2.90±0.09	—		7.36±0.15	—	
SQSTM1+ve						
No	2.15±0.05	-0.659	<0.0001	6.20±0.09	-0.883	<0.0001
Yes	3.47±0.11	—		7.97±0.18	—	
Risk allele category						
Tertile 1	2.61±0.08	-0.200	<0.0001	6.80±0.13	-0.282	<0.0001
Tertile 2	2.91±0.08	0.105	0.025	7.16±0.13	0.080	0.303
Tertile 3	2.90±0.08	—		7.28±0.13	—	
Center						
Florence	2.10±0.09	-0.709	<0.0001	6.72±0.16	-0.365	0.002
PRISM	2.42±0.07	-0.386	<0.0001	5.98±0.12	-1.105	<0.0001
Salamanca	3.38±0.12	0.566	<0.0001	7.57±0.19	0.490	0.001
Naples/Siena	3.00±0.09	0.188	0.009	7.82±0.16	0.740	<0.0001
Western Australia	3.15±0.10	—		7.32±0.18	—	

The data shown are least square means (SD) from a general linear model analysis of predictors of disease extent in the whole study population. The beta-coefficients (Beta) refer to the effect size of the indicated variable on the response variable (no. of affected bones or disease severity score). The *p* values refer to differences between subgroups in each category as compared with the reference subgroup, which is indicated by the (—) symbol.

Table 4.4.: Combined Genetic Risk Score and Markers of Severity in the Whole Study Population.

Variable	Low Risk (n=570)	Medium Risk (n=1190)	High Risk (n=180)	p-value
Number of affected bones	1.74 ± 1.19	2.04 ± 1.48	3.20 ± 2.48	<0.0001
Disease severity score	5.44 ± 2.15	5.86 ± 2.59	7.58 ± 3.67	<0.0001
Severe disease (score >7.0)	146 (25.6%)	378 (31.7%)	99 (55.0%)	<0.0001
Number of previous bisphosphonates	1.12 ± 1.04	1.23 ± 1.00	1.50 ± 1.11	<0.0001
Bone deformity	154 (30.8%)	346 (33.1%)	58 (35.6%)	0.46
Deafness due to Paget's	49 (9.8%)	95 (9.1%)	23 (14.1%)	0.13
Fracture in Pagetic bone	45 (7.9%)	113 (9.5%)	22 (12.2%)	0.20
Orthopaedic surgery	57 (11.4%)	133 (12.7%)	23 (14.1%)	0.59

Values are mean ± SD or number (%). The *p* values refer to the differences between the genotype groups assessed by ANOVA or chi-square test. Those in the low risk group were *SQSTM1* negative and tertile 1 of the risk allele score; the medium risk group *SQSTM1* negative and tertiles 2 to 3 of the risk allele score; and the high-risk group *SQSTM1* positive and tertiles 1 to 3 of the risk allele score.

4.2.5. Role of *SQSTM1* mutations and risk alleles as predictors of clinical severity and treatment response

In view of the fact that the genetic risk score was a strong predictor of disease extent and severity we wanted to determine if we could use this information to identify “high risk” patients and to determine if the response to treatment was influenced by the genetic markers. This analysis was restricted to the PRISM study where we had full information on disease complications including the presence of Pagetic bone pain and prospective data on treatment response. The results are shown in Table 4.5. Although genetic risk predicted severity at baseline, there was no difference in the response to treatment in terms the change in ALP levels or changes in quality of life measures as assessed by the SF36 score according to genetic risk. This illustrates that patients at increased genetic risk of severe disease do not have an impaired response to bisphosphonate therapy.

Table 4.5.: Combined Genetic Risk Score and Markers of Severity in the Whole Study Population.

Variable	Low Risk (n=220)	Medium Risk (n=468)	High Risk (n=82)	p-value
Symptomatic treatment	105 (47.7%)	229 (48.9%)	43 (52.4%)	0.76
Number of affected bones at baseline	1.66 ± 0.92	1.83± 0.97	2.48± 1.22	<0.0001
Disease severity score at baseline	4.85 ± 2.27	4.99 ± 2.29	6.22 ± 2.63	<0.0001
Received bisphosphonate during study	134 (60.9%)	289 (61.7%)	50 (62.2%)	0.97
Total dose risedronate (g)	2.18 ± 3.24	2.00 ± 3.34	2.17 ± 3.64	0.79
Total dose etidronate (g)	0.29 ± 3.12	1.19 ± 8.72	2.98 ± 21.5	0.10
Total dose pamidronate (mg)	45.0± 156	66.9 ± 202.8	39.7 ± 107.5	0.21
Total dose tiludronate (g)	4.94 ± 20.0	6.51 ± 23.5	7.56 ± 28.2	0.60
Change in ALP	-0.34 ± 0.90	-0.32 ± 0.97	-0.39 ± 1.11	0.39
Change in SF36 pain score	-0.53 ± 8.81	+0.12 ± 9.7	-0.88 ± 11.2	0.59
Change in SF36 physical summary	-4.27 ± 9.0	-4.62 ± 9.7	-4.98 ± 10.6	0.86
Change in SF36 mental summary	-1.56 ± 10.1	-2.41 ± 10.8	-3.74 ± 12.1	0.39

Values are mean ± SD or number (%). The *p* values refer to the differences between the genotype groups assessed by ANOVA or chi-square test. The ALP values have been standardized to the upper limit of the reference range, which was set at 1.0.

4.3. Discussion

Genetics factors play an important role in regulating susceptibility to PDB and the findings reported here when combined with other evidence indicate that susceptibility alleles are also associated with disease severity. We previously reported that in the PRISM study, *SQSTM1* mutations were associated with more severe and extensive disease and a higher incidence of certain complications (Visconti et al. 2010). Associations between *SQSTM1* mutations and disease extent have also been observed in other studies (Collet et al. 2007; Hocking et al. 2004). In the present study we have confirmed and extended these observations to show that the risk alleles which we previously found to predispose to PDB by genome wide association study (Albagha et al. 2010; Albagha et al. 2011) are also associated with disease extent and severity.

When we analysed the association between PDB susceptibility alleles and markers of disease in the PRISM study we found that patients in the highest tertile of risk alleles group has significantly more extensive disease and a higher disease severity score as compared with those in the lower two tertiles. The positive association between risk allele score, disease extent and disease severity score was confirmed by meta-analysis of data from independent cohorts in Italy, Spain and Western Australia where we also found that highest tertile group had more extensive disease. In addition we found that in the extended sample, subjects in the highest tertile were more likely to have required previous bisphosphonates therapy than those in the lowest category, consistent with the fact that they had more severe disease.

In all cohorts we found that the new risk alleles acted in an additive manner with *SQSTM1* mutations to regulate disease extent and severity but found that mutations of *SQSTM1* had a quantitatively greater effect than the risk allele score. By combining information from *SQSTM1* status and the new risk alleles however we were able to develop a genetic risk score which delineated three distinct groups with markedly differing effects on disease extent and severity.

Although the genetic risk score was strongly associated with disease extent, there was no difference in response of ALP levels or quality of life according to genetic risk category during

treatment. This is an important observation since it indicates that patients at high genetic risk of severe PDB show no evidence of resistance to the therapeutic effects of bisphosphonates, raising the possibility that complications associated with increased disease severity could be preventable.

In this regard, previous studies have shown that potent bisphosphonates such as Zoledronic acid and Risedronate are highly effective at suppressing bone turnover in PDB (Reid et al. 2005; Singer et al. 1998) and that Zoledronic acid gives prolonged suppression of ALP levels for up to 6.5 years after a single infusion. The effects of bisphosphonate therapy on symptoms of PDB and quality of life are inconsistent however. Several randomised controlled trials of bisphosphonate therapy for PDB have shown a poor correlation between response of ALP and response of bone (Langston et al. 2007b; Miller et al. 1999; Reid et al. 1996). An exception is the HORIZON study where, Zoledronic acid was superior to risedronate at controlling ALP levels and improving some aspects of quality of life in the short term (Reid et al. 2005) and longer term (Reid et al. 2011) . Although this indicates that effective control of elevated bone turnover can give symptomatic improvements, the overall effects of Zoledronic acid on quality of life in HORIZON were modest and the difference between Zoledronic acid and risedronate were less than the 5-point margin which is considered clinically significant. We speculate that these inconsistencies in biochemical and clinical response are attributable to the fact that many patients with PDB have already developed complications before the diagnosis is made and treatment is initiated. This was certainly true in the PRISM study where at baseline, 35% of patients already had bone deformity; 17% had previously required orthopaedic surgery for the disease and 22% had deafness associated with skull involvement.

The results presented here shows that it is possible to predict the severity and extent of PDB by genetic testing for *SQSTM1* mutations and other susceptibility alleles and further research will now be required to define how this information should be beneficially used in clinical practice. One possibility is that patients in the highest genetic risk categories could be prioritised for enhanced surveillance for the development of complications or even prophylactic bisphosphonate therapy. It is important to emphasise however that use of prophylactic bisphosphonate therapy in the absence of symptoms would need to be carefully evaluated to ensure that the benefits of treatment outweigh the potential risks. In this regard it

is relevant to point out that a trial is now in progress, the ZiPP trial (Zoledronate in the Prevention of Paget's disease; ISRCTN11616770) which aims to explore the risk and benefits of prophylactic zoledronic acid therapy versus placebo in patients with *SQSTM1* mutations who have not yet been diagnosed with PDB and it would be of great interest to evaluate the effect of these new markers within this study to determine if they affect penetrance of and severity of the disease in *SQSTM1* mutation carriers.

Chapter 5: Association between Viral Antibodies and PDB

ABSTRACT

Previous studies suggested that PDB may arise as the result of a slow virus infection with one of Paramyxoviridae virus family. It has more recently been proposed that measles virus infection might act as a trigger for disease occurrence and severity in people who are genetically predisposed to develop PDB. Although there is evidence that over-expression of measles virus protein can stimulate osteoclastogenesis and cause PDB-like lesions in mice, attempts to detect viral antigens and nucleic acids from bone and blood samples from affected patients have yielded conflicting results but there have been no previous studies of the immune response to viruses in PDB.

In this chapter circulating antibodies of the IgG class were measured for several viruses in patients with PDB and healthy controls using commercially available enzyme linked immunosorbent assays. Additionally levels of antiviral antibodies were related to markers of disease severity in the PDB cases in relation to other potential determinants of disease severity including age, gender, *SQSTM1* mutation status and allelic score generated by genotypes at other susceptibility loci for PDB.

The case control analysis showed no significant difference in antibody titres directed against rubella, varicella zoster, canine distemper, respiratory syncytial virus or measles in PDB patients as compared with controls. However, circulating antibodies to mumps virus were significantly higher in PDB cases as compared with controls (mean \pm SD = 3.1 ± 0.84 vs 2.62 ± 0.86 , $p < 0.001$), a difference remained significant when corrected for age and gender.

and we study the association between antibody titers and markers of disease severity in PDB cases. There was no significant association between circulating levels of antibody to measles and mumps virus and age of diagnosis, such that subjects in the highest antibody tertile were diagnosed later in life ($p = 0.01$ and $p < 0.0001$ respectively). There was a weak negative association between disease severity score and antibodies to mumps virus ($p = 0.047$) and between number of affected bones and antibodies to distemper virus ($p = 0.049$). There was no evidence of an interaction between predisposing susceptibility alleles for PDB and markers of disease severity for any of the antibodies tested.

These observations suggest that PDB is associated with dysregulation in the antibody response to mumps virus and that antibody responses to mumps and distemper are weakly associated

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with some markers of disease severity. There was no evidence for an interaction between antibody responses to any of the viruses tested and genetic determinants of PDB in predicting disease extent, severity or age at onset. The results are consistent with the hypothesis that PDB may be associated with abnormalities of the immune response but there was no evidence of a specific defect in response to measles virus which has been most implicated in the pathogenesis of the disease.

5.1. Materials and Methods

5.1.1. Patients

The patients studied comprised of patients with PDB who took part in the PRISM study where samples were available from the baseline visit for genetic analysis and for analysis of anti-virus antibodies. Due to the fact that a limited volume of serum was available, antibody testing was prioritised for measles virus antibodies, since this virus has been most implicated in the pathogenesis of PDB and samples for the other viruses were analysed where there was enough serum available for testing. As a result, testing for measles virus antibodies was conducted in 709 patients with PDB as compared with 463 subjects for rubella virus, varicella zoster virus, distemper virus, and respiratory syncytial virus. The controls subjects comprised 220 subjects not known to have PDB who mainly comprised of the spouses of PDB subjects in the PRISM study.

5.1.2. Clinical phenotype

In the PDB patients, information was collected on previous fractures, orthopaedic procedures, use of a hearing aid, bone pain, bone deformity as assessed clinically, gender, age at entry to the study, the age at which PDB was first diagnosed, and the number of affected bones as assessed by radionuclide bone scan. From these data an overall disease severity score was calculated. This is a composite measure of severity taking into account the number of affected bones, and the presence of complications. In summary, one point was assigned for each affected bone and additional points were assigned for the following: previous fractures through Pagetic bone (0=no or 1=yes), previous orthopaedic surgery (0=no or 1=yes), history of osteosarcoma (0=no or 1=yes); bone deformity (0=no deformity; 1=mild or moderate deformity, 2=severe deformity, for each bone affected); use of a hearing aid if the patient had PDB of the skull bones (excluding mandible and maxilla) (0=no or 1=yes); bisphosphonate treatment in the previous 12 months (0=no; 1=yes); bisphosphonate treatment >12 months ago (0=no; 1=yes); and age at diagnosis (1= \geq 70 years; 2=60-69 years; 3=40-59 years; 4=<40 years).

5.1.3. Serum

Serum was extracted from whole blood as described in chapter 2.

5.1.4. Genotyping

Genotyping for mutations of *SQSTM1* was carried out by DNA sequencing of PCR-amplified DNA fragments of exons 7 and 8 and the intron-exon boundaries of *SQSTM1* as described in chapter 3. Genotyping for the other SNP that attained genome wide significance for association with PDB in the studies of Albagha (Albagha et al. 2009; Albagha et al. 2010) was performed either using the Illumina HumanHapDuo300 array; the Sequenom MassARRAY iPLEX platform as previously described, or using TaqMan SNP genotyping assays. A number of randomly selected samples were genotyped on the different genotyping platforms (n=96 on Illumina and Sequenom, n=12 on all three platforms) and the cross-platform genotype concordance rate was 100%.

5.1.5. Measurement of anti-viral antibodies

Testing for immunoglobulin G (IgG) antibodies to viruses was carried out on serum samples collected at the baseline visit using a variety of commercially available enzyme linked immunosorbent assays (ELISA). For measles virus, mumps virus, rubella virus, and varicella zoster virus antibodies the Trinity Biotech Captia assays TM was used (catalogue number 2326000, measles; 2325900, mumps; 2325300, rubella; 2325600, varicella zoster). For respiratory syncytial virus the MP Biomedical RSV IgG ELISA was used (catalogue number 071-516002) and for distemper virus the ImmunoComb® antibody test kit was used. All of the assays were performed according to manufacturer's instructions. Inter-assay and intra-assay CV for each ELISA is available on table 5.1. The reproducibility of the ELISAS is assessed to determine the precision of the test results. Is performed by the manufacturers over 5 sera, run in 20 replicates for intra-assay, five replicates for 3 days for inter-day and 3 replicates of 3 lots for lot to lot. The summary of the results are in table 5.1.

The results of the ELISA assays were read on a Synergy HT Multi-Mode Micro plate reader (Bio-Tek), 450nm filter. Dual wavelength was used and the reference filter set to 600-650nm. All samples were tested in duplicate and the mean value of the two samples calculated. Duplicate samples that yielded values that differed by more than 20% were repeated.

Using a calibrator in each run of ELISA, a mean Optical Density (OD) value is obtained, and to account for day to day fluctuations in the assay performance due to room temperature and timing a Correction Factor is determined by the manufacturers for each lot of kits and printed on the kit; the cut-off calibrator value for each assay is determined by multiplying the correction factor by the mean calibrator OD previously calculated. The Immune Status Ratio (ISR) for each specimen is calculated by dividing the specimen OD value by the cutoff calibrator value determined previously; to transform all the data to International Units (IU) we use the natural exponential function $f(x) = e^x$, where the results will be transformed into IU/ml as indicated by the manufacturer's instructions. Details of the reference ranges for each assay are summarised in Table 5.2. Canine Distemper values are calculated on a different way, based on a colour scale or CombScale provided with the kit, the values range go from S0 to $\geq S5$ which mean High Positive (Fig. 5.1); the comb can be read manually or as done in this case, using CombScan. Comb Scan is a software program that use a computer connected to an scanner; when a Comb is placed on the scanner, the program translate the colour results on the comb into numerical values.

Figure 5.1.: Reading Results with the combscale-Canine Distemper Virus ImmunoComb® ELISA.

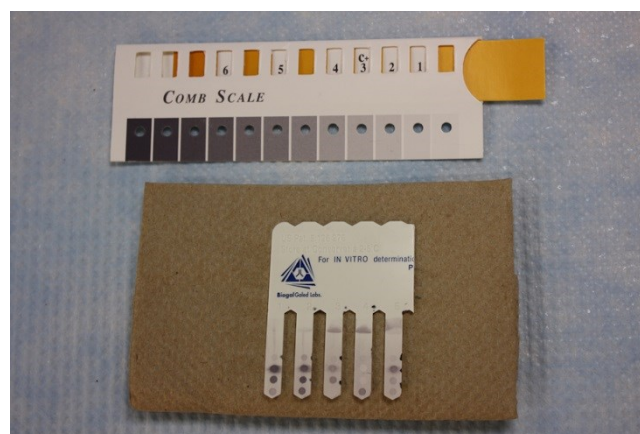


Table 5.1.: Reproducibility; Inter-assay and intra-assay CV for each ELISA, Sensitivity and Specificity given by the manufacturers.

Virus	Intra assay %	Inter assay lot %	Inter assay day %	Relative Sensitivity %	Relative Specificity %
Measles	3.8-17.9	3.7-25.7	4.6-15.2	99.3	91.0
Mumps	4.1-38.1	8.3-14.3	8.3-17.7	99.3	96.6
Rubella	5.1-10.6	4.0-14.5	6.1-16.0	100	97.1
Varicella-Zoster	3.7-18.3	7.0-23.7	6.3-16.3	99.4	97.0
Respiratory Syncytial virus	0.0-6.4	4.8-12.1	0.0-10.7	100	100
Canine distemper	0.0-10	10-30	0-10	90	83

Table 5.2 ELISA kits for viral antibody detection: Reference values and Range. The values are on International Units (IU) with the exception of Canine Distemper.

Virus	Negative	Indeterminate	Positive
Measles	≤ 0.06	0.061-0.09	≥ 0.091
Mumps	≤ 0.9	0.91-1.09	≥ 1.10
Rubella	≤ 6.5	6.6-8.1	≥ 8.2
Varicella-Zoster	≤ 0.11	0.12-0.14	≥ 0.15
Respiratory Syncytial virus	≤ 0.54	0.55-1.09	≥ 1.10
Canine distemper	0	1-2	≥ 3

5.1.6. Statistical analysis

Differences between the levels of antibodies in cases and controls were assessed by analysis of variance when the data were normally distributed or by Mann-Whitney test when the data were not normally distributed. Differences in the proportion of PDB patients and controls that tested positive for antibodies were assessed by the chi-square test. The relation between viral antibody concentrations and markers of disease severity was assessed by analysis of variance after categorising patients into tertiles of low, medium and high antibody levels for each virus. Additional analyses were performed in which the relationship between genotype and disease severity were analysed in relation to circulating viral antibodies. This analysis employed a general linear model analysis of variance, entering genotype, tertile of antiviral antibodies, gender and age into the model with calculation of least square means for the categorical variables. The significance level was set at 0.008 to account for the fact that antibodies against six different viruses were tested.

5.2. Results

5.2.1 Demographic characteristics of PDB cases and controls

The characteristics of PDB cases and controls are summarised in Table 5.3. The proportion of males was higher in PDB cases (53.4% vs 40.0%, $p < 0.001$) and the cases were slightly older than the controls (73.1 vs 71.1 years, $p = 0.002$).

Table 5.3. Demographic characteristics in cases and controls

	PDB (n=709)	Control (n=220)	<i>p</i> -value
Age	73.1 \pm 8.0	71.1 \pm 9.2	$p = 0.002$
Male	379 (53.4%)	88 (40%)	χ^2 , df=1, $p < 0.001$
Age at diagnosis	64.4 \pm 0.4		
Number of affected bones	1.83 \pm 0.04		
Disease Severity Score	5.79 \pm 0.09		

Values are mean SD or number (%). The *p*-value for the difference between groups was assessed by ANOVA for continuous variables or chi-square test for categorical variables.

5.2.2 Qualitative testing for viral antibodies in cases and controls

The results of qualitative testing for the presence or absence of anti-viral antibodies in cases and controls are summarised in Table 5.4. For this analysis patients were categorised as testing, negative, equivocal or positive for anti-viral antibodies according to the manufacturer's reference range (Table 5.2). This analysis showed that a very high proportion of PDB cases and controls tested positive for measles virus, rubella, VZV, mumps and RSV. Antibodies were also detected that cross-reacted with distemper virus in PDB cases and controls but less than 5% were strongly positive. The only difference between cases and controls were for RSV where 91.7% of PDB cases tested positive compared with 97.7% of controls.

5.4 Qualitative testing for viral antibodies in PDB cases and controls

	PDB	Control	Significance
Measles	n=720	n=220	
<i>Negative</i>	2 (0.3%)	2 (0.9%)	
<i>Equivocal</i>	5 (0.7%)	2 (0.9%)	$\chi^2 1.04, df=1, p=0.31$
<i>Positive</i>	216 (99.0%)	216 (98.2%)	
Rubella	n=463	n=202	
<i>Negative</i>	1 (0.3%)	1 (0.5%)	
<i>Equivocal</i>	11 (0.7%)	8 (4.0%)	$\chi^2 1.59, df=1, p=0.20$
<i>Positive</i>	451 (97.4%)	193 (95.4%)	
VZV			
<i>Negative</i>	4 (0.9%)	1 (0.5%)	
<i>Equivocal</i>	11 (2.4%)	2 (1.0%)	$\chi^2 1.68, df=2, p=0.43$
<i>Positive</i>	448 (96.8%)	199 (98.5%)	
Mumps			
<i>Negative</i>	8 (1.7%)	3 (1.5%)	
<i>Equivocal</i>	4 (0.9%)	5 (2.5%)	$\chi^2 2.77, df=2, p=0.25$
<i>Positive</i>	451 (97.4%)	194 (96.0%)	
Distemper			
<i>Negative</i>	2 (0.4%)	3 (1.5%)	
<i>Weak positive</i>	252 (54.5%)	101 (50.0%)	$\chi^2 3.57, df=2, p=0.31$
<i>Positive</i>	194 (41.2%)	89 (44.1%)	
<i>Strong positive</i>	14 (3.0%)	9 (4.5%)	
RSV			
<i>Negative</i>	30 (6.4%)	6 (2.9%)	
<i>Equivocal</i>	8 (1.7%)	0 (0%)	$\chi^2 6.23, df=1, p=0.01$
<i>Positive</i>	432 (91.9%)	199 (97.1%)	

Differences in the proportions of patients in each category were assessed by chi-square test. Where necessary, data were combined for categories where the numbers of observations were too small to provide a valid chi-square statistic.

5.2.3. Antibody concentrations in PDB cases and controls.

The serum levels of anti-virus antibodies in cases and controls were then compared are summarised in Table 5.5. The only significant difference between antibody titres in cases and controls was for mumps virus when levels were significantly higher in PDB cases.

Table 5.5. Levels of antiviral antibodies in PDB cases and controls

Virus	PDB	Control	<i>p</i> -value
Measles	(n=720) 2.38 ± 10.9	(n=219) 1.62 ± 4.4	0.34
Rubella	(n=463) 29.0 ± 11.4	(n=202) 28.3 ± 10.6	0.42
VZV	(n=463) 0.87 ± 0.72	(n=202) 0.85 ± 0.89	0.78
Mumps	(n=463) 3.00 ± 0.84	(n=202) 2.62 ± 0.85	<0.0001
Distemper	(n=462) 2.36 ± 1.08	(n=202) 2.48 ± 1.14	0.20
RSV	(n=470) 226.7 ± 143.4	(n=205) 243.3 ± 137.4	0.16

The values shown are mean ± SD. The number of observations for each virus in cases and controls are indicated. The *p*-values refer to the differences between groups assessed by ANOVA

Since the gender distribution and age of cases and controls differed significantly (Table 5.3), the case control analysis was repeated using GLM ANOVA to adjust for the

differences between cases and controls. The results are summarised in Table 5.6. After adjusting for differences in age and gender the only significant difference between cases and controls was for mumps virus.

Table 5.6 Age and gender adjusted levels of antiviral antibodies in PDB cases and controls

Virus	PDB	Control	<i>p</i> -value
Measles	(n=720) 2.37 ± 0.37	(n=219) 1.72 ± 0.67	0.40
Rubella	(n=463) 29.1 ± 0.52	(n=202) 28.1 ± 0.79	0.38
VZV	(n=463) 0.87 ± 0.05	(n=202) 0.87 ± 0.04	0.96
Mumps	(n=463) 3.00 ± 0.04	(n=202) 2.64 ± 0.06	<0.0001
Distemper	(n=462) 2.36 ± 0.05	(n=202) 2.51 ± 0.08	0.11
RSV	(n=470) 226.6 ± 6.5	(n=205) 244.8 ± 10.0	0.13

The values shown are least square means adjusted for age and gender ± SD. The number of observations for each virus in cases and controls are indicated. The *p*-values refer to the differences between groups assessed by GLM-ANOVA.

5.2.3 Relation between viral antibodies and severity of PDB

For this analysis patients were divided into tertiles on the basis of circulating levels of viral antibodies and antibody levels were related to age at diagnosis, number of affected bones and overall disease severity score which takes account of the number of affected bones and complications of the disease (section 5.1.2). The associations are summarised separately for each virus in Tables 5.7 to 5.12.

Table 5.7. Markers of disease severity in relation to measles virus antibodies

	Antibody Tertile			
	Low	Middle	High	p-value
Number	234	237	238	
Antibody level	0.2 ± 0.08	0.5 ± 0.13	6.3 ± 18.3	<0.0001
Age at diagnosis	64.5 ± 10.8	63.5 ± 11.4	65.1 ± 10.0	0.26
Affected bones	1.8 ± 1.0	1.8 ± 1.0	1.8 ± 1.1	0.95
Total Severity score	5.8 ± 2.6	5.7 ± 2.4	5.8 ± 2.5	0.92

Values are means ± SD. Differences between groups as assessed by ANOVA are indicated

Table 5.8. Markers of disease severity in relation to rubella virus antibodies

	Antibody Tertile			
	Low	Middle	High	p-value
Number	153	154	156	
Antibody level	17.6 ± 5.44	27.7 ± 2.51	41.7 ± 7.9	<0.0001
Age at diagnosis	63.7 ± 11.3	64.3 ± 10.6	64.8 ± 9.8	0.65
Affected bones	1.9 ± 1.1	1.8 ± 1.1	1.7 ± 0.9	0.12
Total Severity score	5.9 ± 2.8	5.7 ± 2.3	5.7 ± 2.2	0.84

Values are means ± SD. Differences between groups as assessed by ANOVA are indicated

Table 5.9. Markers of disease severity in relation to varicella zoster virus antibodies

	Antibody Tertile			
	Low	Middle	High	p-value
Number	157	152	154	
Antibody level	0.3 ± 0.1	0.7 ± 0.1	1.6 ± 0.8	<0.0001
Age at diagnosis	63.2 ± 11.4	64.8 ± 10.1	64.8 ± 10.0	0.27
Affected bones	1.7 ± 0.9	1.8 ± 1.0	1.9 ± 1.1	0.35
Total Severity score	5.6 ± 2.3	5.7 ± 2.4	5.9 ± 2.7	0.47

Values are means ± SD. Differences between groups as assessed by ANOVA are indicated

Table 5.10. Markers of disease severity in relation to mumps virus antibodies

	Antibody Tertile			
	Low	Middle	High	<i>p</i> -value
Number	153	156	154	
Antibody level	0.3 ± 0.1	0.7 ± 0.1	1.6 ± 0.8	<0.0001
Age at diagnosis	64.2 ± 10.3	63.7 ± 11.1	64.9 ± 10.3	0.55
Affected bones	1.7 ± 1.0	1.9 ± 1.1	1.7 ± 0.9	0.34
Total Severity score	5.4 ± 2.2	6.3 ± 2.8	5.5 ± 2.2	0.002

Values are means ± SD. Differences between groups as assessed by ANOVA are indicated

Table 5.11. Markers of disease severity in relation to distemper virus antibodies

	Antibody Tertile*			
	Low	Middle	High	<i>p</i> -value
Number	115	139	208	
Antibody level	0.98 ± 0.1	2 ± 0.0	3.4 ± 0.6	<0.0001
Age at diagnosis	62.6 ± 10.3	64.0 ± 10.1	65.3 ± 10.8	0.087
Affected bones	1.7 ± 0.9	1.9 ± 1.1	1.7 ± 1.0	0.156
Total Severity score	6.0 ± 2.7	5.9 ± 2.5	5.6 ± 2.3	0.229

Values are means ± SD. Differences between groups as assessed by ANOVA are indicated. * It was not possible to categorise patients into tertiles for distemper virus due to the semi-quantitative nature of the assay.

Table 5.12. Markers of disease severity in relation to respiratory syncytial virus antibodies

	Antibody Tertile			<i>p</i> -value
	Low	Middle	High	
Number	157	156	157	
Antibody level	103.6±63.2	195.0±13.2	381.1±131.8	<0.0001
Age at diagnosis	63.9±11.0	64.5±10.7	64.5±10.1	0.88
Affected bones	1.7±1.0	1.9±1.0	1.8±1.1	0.25
Total Severity score	5.7±2.4	5.7±2.5	6.0±2.6	0.37

Values are means ± SD. Differences between groups as assessed by ANOVA are indicated

5.2.4 Interaction between SQSTM1 mutations, viral antibodies and severity of PDB

Since it has been suggested that viral infection might trigger PDB in patients who have SQSTM1 mutations, the relation between SQSTM1 mutations, viral antibody titres and markers of PDB severity were studied. For this analysis patients were categorised by presence or absence of SQSTM1 mutations; the concentrations of antibody to each virus, in tertiles as summarised in 5.2.3, gender and age at entry to the PRISM study.

Measles virus

Measles virus levels were nominally associated with age at onset in that patients in the highest tertile were diagnosed about 3 years older than those in the lower tertile ($p=0.01$) (table 5.5, table 5.6 and table 5.7). However this was not significant taking into account the Bonferroni adjusted threshold of significance of 0.008. There was no significant interaction between measles virus and *SQSTM1* mutations for age at diagnosis ($F=2.44$, $p=0.08$), number of affected bones ($F=0.32$, $p=0.72$) or disease severity score ($F=0.62$, $p=0.54$). However, as previously reported in chapter 3, *SQSTM1* mutation status was a significant predictors of age at diagnosis (64.8 ± 0.4 vs 58.6 ± 1.3 , $p<0.0001$), number of affected bones (2.41 ± 0.2 vs 1.74 ± 0.5 $p<0.0001$)

and disease severity score (7.03 ± 0.2 vs 5.67 ± 0.2 , $p < 0.0001$). Age at entry to PRISM also was associated with age at diagnosis ($F=460.8$, $p < 0.0001$), with number of affected bones ($F=18.54$, $p=0.002$) and with total disease severity score ($F=29.6$, $p=0.028$). Gender was not significantly associated with age at diagnosis, number of affected bones and disease severity score (data not shown).

Table 5.13. Measles virus antibody levels, *SQSTM1* mutations, age, gender and the severity of Paget's disease.

	Antibody Tertile			
Measles	Low	Middle	High	<i>p</i> -value
Age at diagnosis	61.4 ± 0.90	60.9 ± 0.92	64.2 ± 0.80	0.01
Number of affected bones	2.1 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	0.71
Disease Severity score	6.5 ± 0.3	6.1 ± 0.3	6.2 ± 0.2	0.53

Values are least square means \pm SD. Differences between groups as assessed by GLM ANOVA are indicated.

Rubella virus

Rubella virus levels were not associated with any marker of disease severity and there was no significant interaction between rubella viral antibody titres and *SQSTM1* mutations in predicting age at diagnosis ($F=0.28$, $p=0.758$), number of affected bones ($F=0.67$, $p=0.51$) or disease severity score ($F=0.04$, $p=0.958$). As with the measles virus analysis, *SQSTM1* mutation status was a significant predictors of age at diagnosis (65.8 ± 9.6 vs 56.55 ± 9.47 $p < 0.0001$), number of affected bones (1.76 ± 0.96 vs 2.52 ± 1.2 $p < 0.0001$) and disease severity score (5.6 ± 2.3 vs 7.4 ± 2.8 $p=0.001$) after adjusting for antibody titre. Age at entry to PRISM also was associated with age at diagnosis ($F=17.44$, $p < 0.0001$), with number of affected bones ($F=5.52$, $p < 0.0001$) and with total disease severity score ($F=4.25$, $p < 0.001$). Gender was not significantly associated with age at diagnosis, number of affected bones and disease severity score (data not shown).

Table 5.14. Rubella virus antibody levels, *SQSTM1* mutations, age, gender and the severity of Paget's disease.

	Antibody Tertile			<i>p</i> -value
	Low	Middle	High	
Age at diagnosis	61.87±1.36	61.11±1.07	62.23±1.07	0.76
Number of affected bones	2.19±0.17	2.02±0.14	2.01±0.14	0.68
Disease Severity score	6.55±0.42	6.28±0.33	6.24±0.33	0.83

Values are least square means ± SD. Differences between groups as assessed by GLM ANOVA are indicated.

Varicella Zoster Virus

The results are shown in Table 5.15. Varicella zoster virus levels were not associated with any marker of disease severity and there was no significant interaction between rubella viral antibody titres and *SQSTM1* mutations in predicting age at diagnosis ($F=0.34$, $p=0.71$), number of affected bones ($F=0.09$, $p=0.92$) or disease severity score ($F=0.45$, $p=0.64$). As with the measles virus analysis, *SQSTM1* mutation status was a significant predictors of age at diagnosis (64.8 ± 0.4 vs 58.5 ± 1.3 , $p<0.0001$), number of affected bones (2.39 ± 0.2 vs 1.74 ± 0.05 $p<0.0001$) and disease severity score (7.01 ± 0.4 vs 5.68 ± 0.1 , $p<0.0001$) after adjusting for antibody titre. Age at entry to PRISM also was associated with age at diagnosis ($F=329.3$, $p<0.0001$), with number of affected bones ($F=14.12$, $p<0.0001$) and with total disease severity score ($F=10.5$, $p=0.001$). Gender was not significantly associated with age at diagnosis, number of affected bones and disease severity score (data not shown).

Table 5.15. Varicella zoster virus antibody levels, *SQSTM1* mutations, age, gender and severity of Paget’s disease.

	Antibody Tertile			
	Low	Middle	High	<i>p</i> -value
Age at diagnosis	61.43±1.11	61.54±1.19	61.97±1.15	0.94
Number of affected bones	1.96±0.14	2.15±0.15	2.10±0.15	0.66
Disease Severity score	6.05±0.34	6.54±0.37	6.44±0.35	0.57

Values are least square means ± SD. Differences between groups as assessed by GLM ANOVA are indicated.

Mumps virus

Mumps virus antibody levels were significantly associated with age at diagnosis ($p<0.001$) and weakly with total disease severity score ($p=0.047$) but not with number of affected bones. Since age at diagnosis forms a component of the total disease severity score it seems likely that the greater age at diagnosis in the group with the highest tertile of mumps virus antibodies may have been driving this association. There was a significant interaction between mumps viral antibody titres and *SQSTM1* mutations in predicting age at diagnosis ($F=9.46$, $p<0.001$). The least square means for age at diagnosis for the different combinations in Table 5.16. This shows that patients with the highest levels of mumps virus antibodies who are *SQSTM1* positive have an increased age at onset when compared with the lower tertiles suggesting of a gene – antibody response interaction.

Table 5.16. Mumps Virus Least Squares Means for Age of Diagnosis.

	SQSTM1 negative	SQSTM1 positive
Mumps tertile 1	65.1 ± 0.6	52.8 ± 2.3
Mumps tertile 2	64.4 ± 0.6	54.6 ± 2.2
Mumps tertile 3	64.9 ± 0.7	65.3 ± 1.9

However there was no interaction for the number of affected bones ($F=0.18$, $p=0.83$) or disease severity score ($F=12.67$, $p=0.047$). As with the measles virus analysis, SQSTM1 mutation status was a significant predictors of age at diagnosis (64.8 ± 0.4 vs 58.5 ± 1.3 , $p<0.0001$), number of affected bones (2.41 ± 0.2 vs 1.74 ± 0.5 $p<0.0001$) and disease severity score (7.12 ± 0.4 vs 5.67 ± 0.1 , $p<0.0001$) after adjusting for antibody titre. Age at entry to PRISM also was associated with age at diagnosis ($F=337.34$, $p<0.0001$), with number of affected bones ($F=8.41$, $p=0.004$) and with total disease severity score ($F=3.89$, $p=0.049$). Gender was not significantly associated with age at diagnosis, number of affected bones and disease severity score (data not shown).

Table 5.17. Mumps virus antibody levels, *SQSTM1* mutations, age, gender and severity of Paget's disease.

	Antibody Tertile			<i>p</i> -value
	Low	Middle	High	
Age at diagnosis	59.01±1.21	59.51±1.17	65.09±1.02	0.0001
Number of affected bones	2.12±0.16	2.09±0.15	2.00±0.13	0.83
Disease Severity score	6.58±0.37	6.87±0.36	5.74±0.32	0.047

Values are least square means ± SD. Differences between groups as assessed by GLM ANOVA are indicated.

Distemper virus

Distemper virus levels were nominally associated with number of affected bones in that those with the highest tertile of antibodies had fewer bones affected ($p=0.047$) (table 5.18). However this was not significant taking into account the Bonferroni

adjusted threshold of significance of 0.008. There was no significant interaction between distemper virus and *SQSTM1* mutations for age at diagnosis ($F=1.99$, $p=0.138$), number of affected bones ($F=3.09$, $p=0.05$) or disease severity score ($F=1.44$, $p=0.24$). However, as for the previous analyses *SQSTM1* mutation status was a significant predictors of age at diagnosis (64.8 ± 0.4 vs 58.6 ± 1.3 , $p<0.0001$), number of affected bones (2.41 ± 0.2 vs 1.74 ± 0.5 $p<0.0001$) and disease severity score (7.12 ± 0.4 vs 5.71 ± 0.1 , $p=0.01$). Age at entry to PRISM also was associated with age at diagnosis ($F=323.05$, $p<0.0001$), with number of affected bones ($F=9.09$, $p=0.003$). Gender was not significantly associated with age at diagnosis, number of affected bones and disease severity score (data not shown).

Table 5.18. Distemper virus antibody levels, *SQSTM1* mutations, age, gender and the severity of Paget's disease.

	Antibody Tertile			
Measles	Low	Middle	High	p-value
Age at diagnosis	61.4 ± 0.90	60.9 ± 0.92	64.2 ± 0.80	0.01
Number of affected bones	2.1 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	0.71
Disease Severity score	6.5 ± 0.3	6.1 ± 0.3	6.2 ± 0.2	0.53

Values are least square means \pm SD. Differences between groups as assessed by GLM ANOVA are indicated.

Respiratory syncytial virus

Respiratory syncytial virus (RSV) levels were not associated with any marker of disease severity and there was no significant interaction between RSV antibody titres and *SQSTM1* mutations in predicting age at diagnosis ($F=1.03$, $p=0.358$), number of affected bones ($F=0.52$, $p=0.59$) or disease severity score ($F=0.77$, $p=0.46$). As with the measles virus analysis, *SQSTM1* mutation status was a significant predictors of age at diagnosis (64.8 ± 0.4 vs 58.4 ± 1.3 , $p<0.0001$), number of affected bones (2.37 ± 0.2 vs 1.75 ± 0.04 $p<0.0001$) and disease severity score (6.92 ± 0.2 vs 5.69 ± 0.2 , $p=0.03$) after adjusting for antibody titre. Age at entry to PRISM also was associated

with age at diagnosis ($F=358.7$, $p<0.0001$), with number of affected bones ($F=9.37$, $p=0.002$) and with total disease severity score ($F=4.39$, $p=0.03$). Gender was not significantly associated with age at diagnosis, number of affected bones and disease severity score (data not shown).

Table 5.19. Respiratory syncytial virus antibody levels, *SQSTM1* mutations, age, gender and the severity of Paget's disease.

	Antibody Tertile			
Measles	Low	Middle	High	p-value
Age at diagnosis	60.64±1.28	61.32±1.1	62.89±1.06	0.358
Number of affected bones	1.98±0.17	2.09±0.14	2.18±0.13	0.593
Disease Severity score	6.07±0.40	6.19±0.34	6.66±0.33	0.462

Values are least square means ± SD. Differences between groups as assessed by GLM ANOVA are indicated.

5.3. Discussion

The aim of this study was to determine if antibody response to measles virus, RSV and distemper virus which have been implicated in the pathogenesis of PDB (Basle et al. 1985; Friedrichs et al. 2002; Gordon et al. 1993; Hoyland et al. 2003; Mills et al. 1981; Ralston et al. 1997) was associated with disease occurrence or severity. In addition to testing these viruses I also measured antibody titres to other viruses such as mumps, rubella and varicella zoster virus which have not been implicated in the pathogenesis of Paget's disease.

The results showed that a very high proportion of patients in both the PDB group and controls had circulating antibodies to the human viruses tested. In most cases the prevalence of those that tested positive was greater than 95% with only a small number of individuals that tested negative or where the results were intermediate. For distemper virus about 50% tested weakly positive, with 40% positive and only 5% strongly positive. Since there is homology between distemper virus and other members of the paramyxovirus family (Kallajoki et al. 1991; Orvell and Norrby 1980; Sheshberadaran et al. 1986), it's unclear if these antibodies truly represented previous distemper virus infection or whether antibodies within the patient's serum were cross-reacting with the distemper ELISA. Further studies will be required to assess this.

On qualitative testing there was no significant difference between cases and controls in the proportion of those that tested positive for any of the viruses studied except RSV where a greater proportion of PDB patients tested negative for antibodies than controls (6.4% vs 2.69%, $p=0.01$).

On quantitative testing the only difference between groups was observed for mumps virus. In this case PDB patients had a significantly higher titre of antibodies than controls (3.0 ± 0.8 vs 2.6 ± 0.8 , $p<0.001$).

Another aim of the study was to determine if the levels of virus antibody were related to markers of severity of PDB. For this analysis patients were divided into three groups on the basis of viral antibody titre and this was related to age at diagnosis, number of affected bones and the overall disease severity score which takes into account the number of affected bones, along with other complications of the disease and age at onset. The only significant difference that was observed was in relation to mumps virus where the overall disease severity score was related to mumps antibody level. Specifically subjects with mumps virus titres in the middle tertile had higher severity than the other two tertiles (6.3 ± 2.8 , vs 5.4 ± 2.1 and 5.5 ± 2.3

respectively). However this was at the borderline of significance ($p=0.002$) and could have represented a false positive result.

Given that genetic factors play an important role in PDB a further analysis was conducted to determine if SQSTM1 mutation status and viral antibody titres interacted to influence severity. This analysis showed that SQSTM1 mutations strongly predicted age at onset, number of affected bones and overall disease severity score. However we found little evidence of an interaction between viral antibody titres and SQSTM1 in predicting disease severity with the notable exception of mumps virus where subjects with the highest levels of antibodies that were SQSTM1 positive had an increased age at diagnosis than the other genotype / viral antibody groups.

Overall the studies do provide no support for the notion that patients with PDB have an abnormal antibody response to paramyxovirus or have had previous infections with these viruses more frequently than controls. This of course does not exclude the possibility that PDB patients might have a clinically occult slow virus infection which is not accompanied by an abnormality in the immune response. Having said that, subacute sclerosing pan-encephalitis, the archetypical slow virus infection with measles is associated with abnormal immune response with increased levels of antibody production in blood ($\geq 1:256$) and the CSF ($\geq 1:4$) (Garg 2002).

Interestingly evidence for an abnormality in the immune response to mumps virus was detected both in respect to the occurrence of PDB and disease severity score. Given that greater disease severity was only detected in participants within the middle tertile of mumps virus antibodies it is possible that this may represent a false positive result. Previous studies have shown evidence to suggest that immunoglobulin levels are abnormal in PDB compared with controls, although this study was small (Buxbaum and Kammerman 1984). This raises the possibility that PDB might be associated with subtle abnormalities in the immune response which would not be surprising given that many of the genes implicated in the pathogenesis of PDB such as RANK, OPG and OPTN also play a role in regulating immune function (Bengtsson and Ryan 2002; Boyce and Xing 2007; Mori et al. 2013; Walsh and Choi 2014). Further studies would be of interest to determine if the abnormal antibody production to mumps virus that was observed here is a feature specific to mumps or might represent a wider abnormality of the immune response in patients with PDB.

Chapter 6: Future Work

For a long time PDB has been thought to have a genetic cause due to the observation of a pattern of familial transmission in many studies (Eekhoff et al. 2004a; Hocking et al. 2000; Laurin et al. 2001; Morissette et al. 2006). Additionally, linkage studies have identified a number of chromosomal regions which are linked to disease in such families (Good et al. 2001; Hocking et al. 2001; Laurin et al. 2001). In chapter 3, genotyping the prism population, exon 7 and exon 8, for *SQSTM1* mutations, allowed us to identify common mutations and even identify some novel mutations which also open the possibility of the **future study of novel mutations, structural and functional studies as well as the opportunity of using animal models**. This could be achieved by: studying and defining the complexity of (poly) ubiquitin modifications *in vivo* by the application of proteomics-based methods; also creating robust animal models carrying the mutations, will allow fully described and understand the genetics consequences of the mutations and possibly to provide a high-throughput genetically modifiable platform for preclinical evaluation of new therapeutics.

Additionally we related the presence of the mutations with the severity of the disease and found that cases with the mutations on *SQSTM1* have an earlier age of diagnosis, more bones affected, are more likely to have orthopaedic surgery, more often to have pagetic bones fractures and in general a more severe presentation of the disease than the patients without the mutation.

Mutations in *SQSTM1* gene are found in around a third of families with PDB, and 10 to 11% of sporadic cases. There is a high percentage of cases where *SQSTM1* mutations are not present. Other potential loci linked to the disease have also been identified and are currently under study. In Chapter 4 we mention the identification of novel genetic markers that predict disease severity and complications in PDB. Genetics factors play an important role in regulating susceptibility to PDB and the findings reported in

Chapter 4, when combined with other evidence, indicate that susceptibility alleles are also associated with disease severity. Associations between *SQSTM1* mutations and disease extent have also been observed in other studies (Collet et al. 2007; Hocking et al. 2004). In the present study we have confirmed and extended these observations to show that the risk alleles which we previously found to predispose to PDB by genome wide association study (Albagha et al. 2010; Albagha et al. 2011) are also associated with disease extent and severity. More work has already been published recently, extending the dimension of this study (Albagha et al. 2013; Obaid et al. 2011).

A number of environmental factors have been suggested to be involved in the disease. Changes in severity and occurrence of the disease have been found together with variable penetrance of Paget's disease within families with a genetic predisposition, demonstrating that an environmental factor may be operative. The concentration of cases in Lancashire, England (Barker et al. 1980) and in Australia (Gardner et al. 1978) strongly suggests an important environmental factor in the aetiology. However, the focal nature of Paget's is still unexplained, in the fact that the disease remains highly localized to a particular bone or bones rather than affecting the whole skeleton. The leading hypothesis for an infectious aetiology in Paget's disease of bone is the **slow virus infection** theory which we explored on Chapter 5.

In chapter 5, I discussed the study of a variety of virus. The data showed that Rubella, RSV, Distemper, VZV and Measles IgG levels are not different between cases and controls; but what is still interesting are the levels of antibodies found against specific virus. Additionally, we found that there is a significant difference between cases and controls with regard to IgG levels specific to Mumps virus, where the people with PDB showed higher levels than the controls. Also in Chapter 5, studying further into the cases, I found that for Mumps virus IgG levels, the cases are diagnosed earlier, suffered from deafness and have a higher risk of fractures if the levels of IgG against Mumps are higher or positive. Cases with negative levels or IgG against Mumps or non-

protective levels did not have records of pagetic fractures. If PDB is related to a viral infection, this study corroborated the fact that it may be Mumps virus, not measles or other paramixovirus. **However, further studies will be required to clearly establish the relationship between PDB and MUMPS virus such as analysis of local Pagetic lesions and RT-PCR for specific viral particles in pagetic cases.**

It has been reported previously, that bisphosphonates such as Pamidronate may cause reduction in serum of IgG levels (Zhang et al. 2007); our data suggested some similar happening when we analysed the levels of IgG against measles in the cases. More recently it has been published that the oral administration of soluble proteins with first generation clodronate, zoledronate and neridronate induced transient specific soluble protein serum IgG (Tonti et al. 2013); recognizing that these drugs may enhance B cell responses in humans (transiently). We used a very small number of cases and controls. A bigger number study will clearly answer this question. Perhaps also studying the production of Immunoglobulins in-vitro in cases and controls will give us an idea of the functioning of the Immune System on the PDB meaning that **a comparative study of the synthesis of Immunoglobulin on PDB cases and controls is required**. Further studies on different bisphosphonates effect on the production of Immunoglobulins and the immune response in general will be also complement and complete the study.

Electronic Database Information

URLs for databases used in this study are as follows:

Human Genome Mapping Project, <http://www.hgmp.mrc.ac.uk/> (software or conducting SimWalk analysis)

National Centre for Biotechnology Information (NCBI), <http://www.ncbi.nlm.nih.gov/> (source of genomic and mRNA sequence information)

Official Rebase Website, The <http://rebase.neb.com> (data base for restriction enzymes which could be use to genotype C1215T)

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (catalogue of human genes and genetic disorders.

As citation:

1. (http://www.euvac.net/graphics/euvac/pdf/annual_2010.pdf).
2. (http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/1195733833790)

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